Carcinogenesis vol.28 no.11 pp.2355–2362, 2007
doi:10.1093/carcin/bgm216
Advance Access publication October 4, 2007

High red meat diets induce greater numbers of colonic DNA double-strand breaks than white meat in rats: attenuation by high-amylose maize starch

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Human population studies show that dietary red and processed, but not white, meats are associated with increased risk of colorectal cancer but dietary fibre appears to be protective. We examined whether dietary cooked red or white meat had differential effects on colonic DNA damage in rats and if resistant starch (RS), a dietary fibre component, provided protection. Rats were fed diets containing approximately 15, 25 or 35% of cooked beef or chicken, both with or without 20% high-amylose maize starch (HAMS) as a source of RS, for 4 weeks. DNA single-strand breaks (SSB) and double-strand breaks (DSB) were measured in isolated colonocytes (by comet assay) along with apoptosis levels, colonic mucus thickness and large bowel short-chain fatty acids (SCFA). Both red and white meat increased colonocyte SSB and DSB dose dependently but damage was substantially greater with red meat. Dietary HAMS prevented these increases. Apoptotic cell numbers were increased dose dependently by red meat irrespective of HAMS feeding, whereas white meat only increased apoptotic cell numbers in the presence of HAMS. Red meat induced greater colonic mucus layer thinning than white meat but HAMS was protective in both cases. HAMS induced increases in large bowel SCFA, including butyrate, and significantly lowered concentrations of phenols and cresols. We have demonstrated that dietary red meat causes greater levels of colonic DNA SSB and DSB than white meat, consistent with the epidemiological data. Dietary RS protects against this damage and also against loss of the mucus barrier, probably through increased butyrate production.

Introduction

Recent epidemiological data indicate an increased risk of colorectal cancer (CRC) through consumption of red or processed meats but not white meat and that dietary fibre is protective (1–5). An international epidemiological study also identified protein *per se* as an independent risk factor for CRC (6). Genetic damage is a prerequisite for cancer and previously we have used a rat model to explore the effects of dietary proteins on colonocyte DNA single-strand breaks (SSB) as measured by the comet assay (7–9). We showed that casein, cooked red meat or soy, but not a whey extract, increased genetic damage. Cooked red meat consumption also resulted in a significant thinning of the colonic mucus barrier. These adverse outcomes induced by high concentrations of protein were largely abolished by the dietary inclusion of high-amylose maize starch (HAMS) as a source of resistant starch (RS). As little as 10% of HAMS in the diet reduced DNA damage induced by a high-casein diet by ∼40% (10). This is consistent with the study of Le Leu *et al.* (11), which shows that similar levels of dietary HAMS are capable of ameliorating dietary protein enhancement of azoxymethane-induced tumorigenesis in rats. Other studies have shown that some diets, including high-meat, high-fat, low-fibre 'western' diets, can increase the toxicity of human faecal water on cultured colon cancer cell lines (12–15). These data indicate that a high level of red meat consumption (in the absence of RS) increases the genotoxic load in the colon and creates an environment conducive to tumorigenesis.

RS is fermented extensively by the large bowel microflora leading to the production of short-chain fatty acids (SCFA). SCFA promote visceral function and are likely mediators of the protection afforded by RS against protein-induced DNA damage. It appears likely that a specific acid, butyrate, is the prime protective agent as it promotes a normal cell phenotype, including enhancing apoptosis in cells with extensive DNA damage (16).

In the present study, we have used rats to determine whether the observed differences in CRC risk in humans for diets high in red and white meats are reflected in differential levels of DNA damage in colonocytes and to determine if dietary RS (as HAMS) was protective. Also for the first time, the effects of high protein and RS diets on numbers of both SSB and double-strand breaks (DSB) in DNA of extracted colonocytes were examined. DSBs are regarded as being a more deleterious form of DNA damage than SSBs in relation to carcinogenesis (17). To determine if effects of protein on bowel health are dose dependent, we have included meat in the diet at 15, 25 and 35%. HAMS was included in the diet at 20%, a level shown previously sufficient to protect against high protein-induced colonic DNA damage (10). Numbers of apoptotic colonocytes, colonic mucus thickness, caecal and faecal SCFA concentrations and weights of various tissues are also reported.

Abbreviations: CRC, colorectal cancer; DSB, double-strand break; HAMS, high-amylose maize starch; RS, resistant starch; SCFA, short-chain fatty acid; SSB, single-strand break.

Materials and methods

Animals and diets

Adult, male Sprague–Dawley rats (n = 96) weighing ∼200 g were obtained from the Animal Resource Centre, Murdoch University, Perth, Western Australia. Rats were housed in wire-bottomed cages in a room of controlled heating and lighting (23°C with a 12 h light–dark cycle) and had free access to food and water. They were allocated randomly to 12 groups (n = 8 per group) and fed one of 12 diets (Table I) for 4 weeks.

The diets, based on the AIN-93 diet (18), contained 15, 25 or 35% red meat (beef round rump boneless steak trimmed of fat) or 13, 22 or 30% white meat (chicken breast trimmed of fat) with or without 20% HAMS (Hi-maize™, National Starch and Chemical Company, New South Wales, Australia). Meats, purchased from Central Market Meat (Adelaide, South Australia, Australia), were cooked on a hotplate with a temperature of 150°C until lightly browned. The meat was then dried at 45°C for 48 h and milled to provide products containing 73.4% protein and 18.3% fat (beef) and 84.7% protein and 8.9% fat (chicken). These values are expressed on a dry weight basis with bound water making up the difference to 100%. To compensate for higher protein levels in the chicken meat, slightly lower doses of chicken meat were chosen to give equivalent levels of protein to those in red meat. Beef tallow was added to the chicken treatments to match the fat content in the beef treatments. Diets without HAMS contained highly digestible starch (cornstarch, National Starch and Chemical Company) instead. The ileal digestibility of HAMS and the cornstarch were previously determined in rats and shown to be 58% (i.e. 42% RS) and 99% (1% RS), respectively (19). All diets contained 5% wheat bran as fibre source.

In the final 4 days of the experiment, rats were placed in metabolic cages to measure faecal and urinary output. At the conclusion of the study, the rats were anesthetized with 4% halothane/oxygen and gut tissues and digesta were collected for analyses. All procedures involving animals were approved by the Commonwealth Scientific and Industrial Research Organisation CSIRO Human Nutrition Animal Ethics Committee and the University of Adelaide Animal Ethics Committee.

Comet assay (single-strand and double-strand DNA breaks)

A 6 cm segment of colon was removed from each rat at a point 3 cm from the most distal end of the colon and colonocytes were isolated immediately.

Table I. Composition of experimental diets (g/kg diet)

	Dietary treatment											
	0% HAMS						20% HAMS					
	Red meat			White meat			Red meat			White meat		
	15%	25%	35%	15%	25%	35%	15%	25%	35%	15%	25%	35%
Red meat	150	250	350	0	0	0	150	250	350	0	0	0
White meat	0	0	0	130	220	300	0	0	0	130	220	300
Cornstarch	580	500	418	585	503	430	380	300	218	385	303	230
HAMS	0	0	0	0	0	0	200	200	200	200	200	200
Sucrose	100	100	100	100	100	100	100	100	100	100	100	100
Sunflower oil	30	30	30	30	30	30	30	30	30	30	30	30
Tallow	40	20	1.5	55	47	40	40	20	1.5	55	47	40
Wheat bran	50	50	50	50	50	50	50	50	50	50	50	50
L-cystine	3	3	3	3	3	3	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Vitamins (AIN-93)	10	10	10	10	10	10	10	10	10	10	10	10
Minerals (AIN-93)	35	35	35	35	35	35	35	35	35	35	35	35

Total protein and fat of white meat groups were matched with corresponding red meat groups. Diets based on AIN-93 formulation.

These cells were used for the measurement of DNA SSB using the single-cell gel electrophoresis (comet) assay as described previously (20). DNA DSBs were also measured using the comet assay as described previously (20). Comet tail moment is the product of tail length and the fraction of DNA in the tail and was calculated for 50 cells from each of three slides per rat. The measure was calculated by Scion Image Beta 4.02 image processing and analysis software (Scion Corporation, Frederick, MD) using a public domain macro (21).

Apoptosis

Apoptosis was measured using a diffusion apoptosis slide halo assay kit (Trevigen, Gaithersburg, MD). Two hundred cells were measured in duplicate for 12 animal groups and nucleoids with halo patterns were counted using a fluorescence microscope (EX41, Olympus, Tokyo, Japan) with Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

Colonic mucus layer thickness

A 1 cm segment of colon was removed from each rat at a point 2 cm from the most distal end of the colon and cut open along the anti-mesenteric ridge and the mucosal surface was washed gently with 0.15 M NaCl solution to remove digesta. The thickness of mucus lining the colon was determined by further cutting the tissue into 1.6 mm lengths, illuminating the tissue segments, capturing numerous images of the mucus layer along each segment, and then measuring the thickness using an image analysis programme as described previously (7). For each animal, 10 measurements were taken at different points along four tissue segments to give 40 thickness measurements in total.

SCFAs and phenols

Faecal and caecal contents were thawed and then distilled and homogenized with 20 µl of 1.68 mM of internal standard (heptanoic acid). The contents were analysed for SCFA (acetic, propionic and butyric acids), in duplicate, using an Agilent Technologies 6890N Network Gas Chromatograph System fitted with a Zebron ZB-FFAP column (0.53 mm × 30 m) (Phenomenex, Torrance, CA) as described previously (22). Caecal and faecal phenol and *p*-cresol were analysed by a previously described method (23).

Statistical analyses

The experiment was designed as a randomized complete block design based on 2 × 6 factorial treatment arrangement [two dietary protein types (three levels) and two RS levels]. Data are presented as the mean ± SEM for each treatment group and presented with pooled SEM in tables. The effect of HAMS and protein and their interactions were determined by three-way analysis of variance and differences between treatments were analysed *post hoc* by Tukey's test. The relationship among caecal and faecal characteristics, colonic DNA damage and mucus layer thickness was determined by Pearson coefficient of regression model controlling for the effect of the different diet. Analyses were performed using a SAS statistical software program (version 8.02 Statistical Analysis System Institute, Cary, NC). A value of $P < 0.05$ was used as the criterion of significance.

Results

Body weight gain and daily food and water intake

The mean initial body weight (Table II) for all groups combined was 200 ± 2 g. Final body weight was unaffected by protein type or level. However, it was lowered significantly in rats fed HAMS compared with those fed the control starch ($P < 0.01$). The mean weekly weight gain was unaffected by protein type or level but was significantly lowered in rats fed HAMS ($P < 0.01$). Food intake was not affected by diets (Table II). There was no effect of protein type or RS on daily water intake, but water intake increased as protein level increased.

Caecal content and tissue weights, faecal and urine output, pH, intestinal weight and length

The wet weight of caecal contents (Table II) was 2.7 times higher in rats fed HAMS than those fed standard starch ($P < 0.001$). Caecal digesta wet weights were significantly higher in rats fed red meat plus HAMS compared with those fed the white meat plus HAMS. Caecal tissue weight was significantly greater in rats fed HAMS but was unaffected by protein type and level.

Faecal output (Table II) was unaffected by protein type but was increased by feeding HAMS. Daily urine output was unaffected by HAMS but rose with increasing dietary protein. Both caecal and faecal pH were lowered significantly by feeding HAMS ($P < 0.0001$).

There was an increase in colon length (Table II) when rats were fed the HAMS compared with those fed the control starch ($P < 0.01$). Rats fed the red meat diets showed significant increase in colon tissue weight compared with those fed the white meat diets ($P < 0.05$). Colonic digesta wet weights were unaffected by diet (Table II). There were no main effects of protein level, protein type or HAMS on small intestinal length or tissue weights (Table II). However, there were significant interactive effects of protein type and HAMS and protein level and HAMS on small intestinal tissue weights.

DNA damage and apoptosis

Colonic DNA SSB (Figure 1A) were increased significantly and dose dependently ($P < 0.001$) with increased dietary protein as red or white meat in rats fed the control starch. This increase was significantly greater for red than for white meat ($P < 0.001$). However, the difference between red and white meat in the absence of RS was only at the highest level of protein. When the diet contained HAMS, there was no significant increase in colonocyte SSB with increased dietary protein nor were there any differences between the meats.

Table II. Effects of dietary red meat, white meat and HAMS levels on body weights, daily food and water intakes, faecal and urinary outputs and caecal, colon and small intestine measurements in rats

	Dietary treatment												Main effects (P values)						
	0% HAMS						20% HAMS						Pooled SEM	HAMS	Protein type	Protein level	Protein type × protein level	Protein type × HAMS	Protein level × HAMS
	Red meat			White meat			Red meat			White meat									
	15%	25%	35%	15%	25%	35%	15%	25%	35%	15%	25%	35%							
Final weight (g)	358^{ab}	355^{ab}	349^{a}	366^{b}	367^{b}	364^{b}	343^{ac}	325^{c}	343^{ac}	330^{c}	342^{ac}	374^{b}	11	<0.01	NS	NS	NS	NS	NS
Mean weight gain (g/week)	41^{ab}	41^{ab}	40^{abc}	41^{ab}	41^{ab}	39^{abc}	36^{bcd}	32^{d}	36^{bcd}	32^{d}	35^{cd}	42^{a}	3	<0.01	NS	NS	NS	NS	NS
Daily food intake (g/day)	18.9	18.5	17.3	18.2	18.3	17.4	18.0	17.6	18.7	17.5	18.1	17.9	0.7	NS	NS	NS	NS	NS	NS
Daily water intake (ml/day)	22.3^{ab}	23.4^{ab}	25.8^{ab}	20.7^{b}	23.8^{ab}	25.4^{ab}	20.2^{b}	23.0^{ab}	33.2^{c}	23.8^{ab}	26.6^{abc}	29.4^{ac}	2	NS	NS	<0.001	NS	NS	NS
Caecal measurements																			
pH	7.19^{ab}	7.01^{a}	7.09^{ab}	7.26^{b}	7.10^{ab}	7.16^{ab}	6.32^{cd}	6.49^{c}	6.24^{d}	6.37^{cd}	6.32^{cd}	6.03^{d}	0.15	<0.0001	NS	NS	NS	NS	NS
Content weight (g)	1.3^{a}	1.6^{a}	1.5^{a}	1.3^{a}	1.4^{a}	1.3^{a}	3.0^{bc}	3.3^{b}	3.4^{b}	2.4^{d}	2.5^{cd}	2.5^{cd}	0.2	<0.001	<0.001	NS	NS	NS	NS
Tissue weight (g)	1.2^{ab}	1.0^{b}	1.2^{abc}	0.9^{b}	1.1^{b}	0.9^{b}	1.6^{cd}	1.7^{d}	1.7^{d}	1.4^{acd}	1.7^{d}	1.8^{d}	0.1	<0.001	NS	NS	NS	NS	NS
Colon measurements																			
Content weight (g)	0.7^{abc}	0.6^{abc}	0.5^{bc}	0.6^{bc}	0.6^{abc}	0.4^{c}	0.6^{abc}	0.6^{abc}	0.5^{bc}	1.0^{a}	0.8^{abc}	0.9^{ab}	0.2	0.06	NS	NS	NS	<0.05	NS
Tissue weight (g)	1.5^{abc}	1.7^{ab}	1.4^{abc}	1.4^{bc}	1.3^{bc}	1.1^{ac}	1.6^{ab}	1.7^{a}	1.4^{abc}	1.4^{abc}	1.4^{abc}	1.5^{ab}	0.1	NS	<0.05	NS	NS	NS	NS
Tissue length (cm)	17.6^{ab}	18.3^{ab}	17.6^{ab}	18.1^{ab}	17.4^{ab}	17.1^{a}	18.7^{ab}	17.6^{ab}	18.9^{b}	19.0^{b}	19.0^{b}	19.1^{b}	0.6	<0.01	NS	NS	NS	NS	NS
Small intestine measurements																			
Tissue weight (g)	8.8^{ab}	8.5^{ab}	8.0^{b}	8.1^{b}	8.2^{b}	7.9^{b}	8.0^{b}	7.9^{b}	8.1^{b}	7.9^{b}	8.3^{ab}	9.3^{a}	0.4	NS	NS	NS	NS	<0.05	<0.05
Tissue length (cm)	114.0^{ab}	115.1^{ab}	112.5^{a}	113.6^{ab}	118.9^{b}	115.9^{ab}	112.6^{a}	112.1^{a}	119.3^{b}	113.8^{ab}	116.0^{ab}	117.0^{ab}	2.1	NS	NS	NS	NS	NS	NS
Faecal measurements																			
pH	6.88^{a}	7.21^{b}	6.93^{a}	7.30^{b}	7.19^{b}	7.11^{b}	6.40^{c}	6.45^{c}	6.52^{c}	6.03^{d}	6.21^{d}	5.88^{d}	0.15	<0.0001	NS	NS	NS	<0.001	NS
Output (g/day)	2.3^{ab}	2.4^{ac}	2.3^{ab}	2.0^{ab}	2.2^{ab}	1.8^{b}	2.8^{cd}	3.2^{def}	3.3^{ef}	2.9^{de}	3.2^{def}	3.6^{f}	0.2	<0.0001	NS	NS	NS	<0.05	<0.01
Urine output (ml/day)	8.2^{abc}	9.4^{abd}	12.3^{de}	6.6^{ac}	8.9^{ab}	10.0^{bde}	5.7^{c}	7.5^{abc}	12.1^{de}	8.4^{abc}	10.3^{bde}	12.9^{e}	1	NS	NS	<0.0001	NS	<0.01	NS

Values are presented as the mean ($n = 8$) and the pooled SEM.
[a–e]Mean values in a row with unlike superscript letters were significantly different ($P < 0.05$).

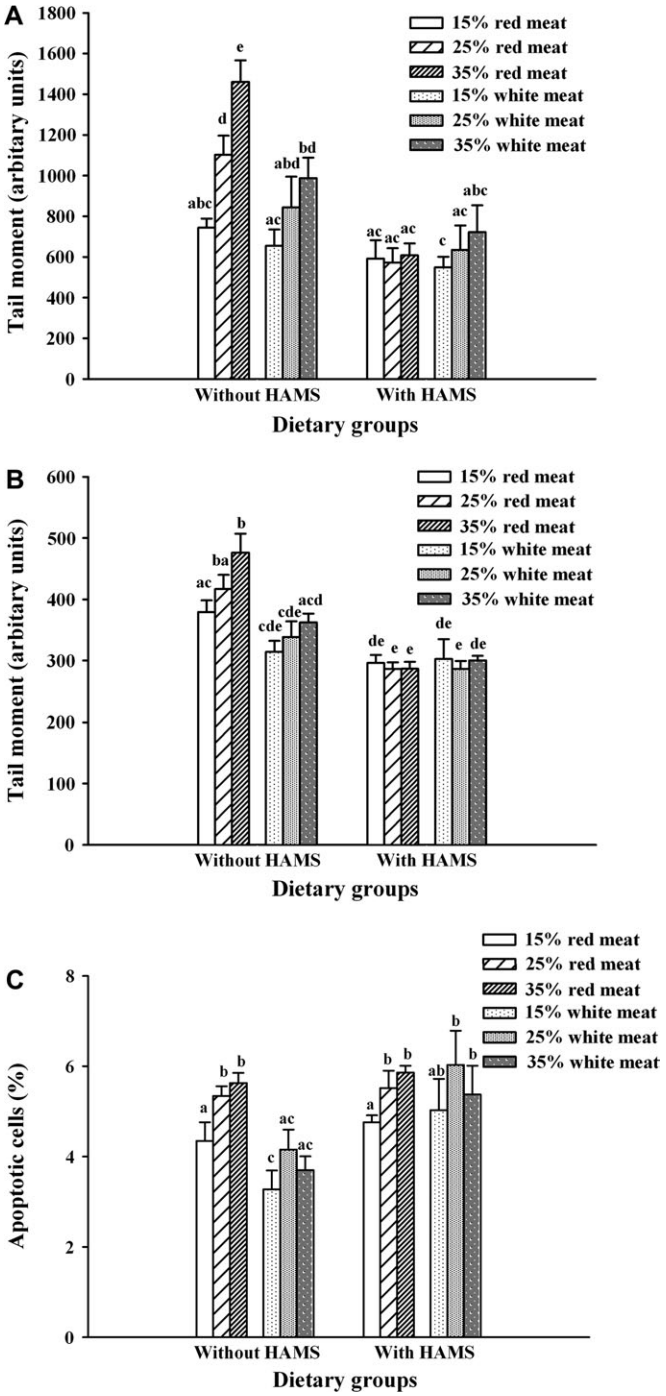

Fig. 1. Effects of dietary cooked red or white meat (at approximately 15, 25 and 35% of diet) with or without 20% high-amylose maize starch (HAMS) on colonic DNA damage (**A** single-strand DNA breaks; **B** double-strand DNA breaks) and apoptosis (**C**) levels in rats. The comet assay was performed on colonocytes extracted from the colon and the resulting comet tail moments (comet tail length x % DNA in the tail) are presented. The % of cells undergoing apoptosis was determined by the halo assay also using colonocytes extracted from the colon. All data are presented as the arithmetic mean ± SEM (n = 8). Values not sharing the same letter are significantly different (**P** < 0.05).

As with SSB, colonocyte DNA DSB rose with greater dietary protein levels in rats fed the control starch (Figure 1B). However, the increased genetic damage was significant only in rats fed the red meat diet. There were significantly greater numbers of DSB with red meat than white meat in rats fed the control starch ($P < 0.0001$) while the feeding of HAMS abolished any effect of dietary protein on this index of genetic damage. There were similar trends in comet tail length as for SSB and DSB based on tail moment as described above (data not shown).

The feeding of red meat diets resulted in a significantly higher number of colonocytes undergoing apoptosis than white meat diets in rats fed the control starch (Figure 1C; $P < 0.001$). However, there was no difference in the effects of the two meats on apoptosis when HAMS was present. The effects of red meat on apoptosis levels were effectively unchanged by the presence or absence of HAMS but when white meat was consumed, apoptosis levels were significantly higher for diets containing HAMS compared with diets without HAMS ($P < 0.001$). Consequently, there was a significant overall increase in apoptosis with dietary HAMS ($P < 0.05$).

Colonic mucus layer thickness

Colonic mucus layer thickness was thicker in all rats fed HAMS compared with those fed standard starch (Figure 2; $P < 0.001$). A significant dose-dependent loss of mucus barrier thickness was seen in rats fed the red meat diet plus the control starch but not in the corresponding groups fed white meat. Regression analysis indicated an inverse correlation between comet tail moment of colonocytes and mucus thickness (SSB: $n = 96$, $r = -0.36$, $P < 0.001$; DSB: $r = -0.43$, $P < 0.0001$).

Caecal and faecal SCFA

Acetate, propionate, butyrate and total SCFA pools were measured in the caecum and faeces and all were significantly increased by dietary HAMS (Table III; all $P < 0.0001$). There was a significant independent effect of protein type on caecal acetate, propionate and total caecal SCFA pools with higher values overall in rats fed red meat plus HAMS compared with those fed white meat. The faecal butyrate pool was significantly higher in rats fed white meat than in those fed red meat. In contrast, dietary red meat increased the caecal butyrate pool compared with white meat. Regression analysis indicated that there was a significant correlation between caecal butyrate pools and apoptosis level ($n = 96$, $r = 0.35$, $P < 0.001$), but no significant relationship was found for the other SCFA.

Phenol and p-cresol

There was a significant reduction in both caecal and faecal concentrations of phenol and *p*-cresol for rats fed HAMS compared with those fed without HAMS (Table IV). Caecal and faecal *p*-cresol concentrations, but not phenol concentrations, were increased to a greater extent by red meat than white meat overall.

Discussion

In this study, we showed, in rats, that dietary red meat results in significantly greater number of colonic DNA SSB and DSB than white meat as measured by the comet assay. Genetic damage is a prerequisite for cancer and so these data are consistent with the epidemiological findings linking higher consumption of red meat to greater CRC risk. The present data are consistent also with the absence of excess risk with white meat consumption noted in those population surveys. This extends our previous studies showing that high levels of dietary protein from a range of sources, including red meat, can increase colonic DNA SSB and that dietary RS inclusion is protective (8). In particular, we have now measured DSB, a form of DNA damage linked more closely to risk of carcinogenesis (24,25), and have demonstrated a dose-dependent increase in these breaks in response to red meat. As in our previous studies, the protection afforded by dietary RS in the present study relates well with increases in the production of the SCFA, butyrate in particular, which is thought to play a role in maintaining the integrity of colonic cells.

DNA damage in somatic cells of eukaryotes can take the form of SSB, DSB or DNA adducts (covalent modifications of DNA bases) and, unless such damage is repaired, the retained errors can ultimately

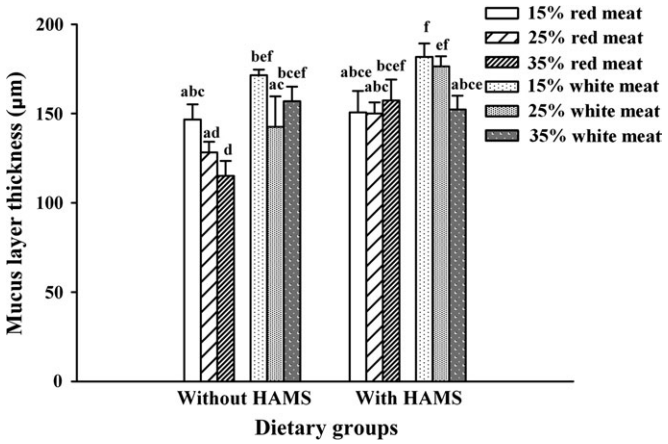

Fig. 2. Effects of dietary cooked red or white meat (at approximately 15, 25 and 35% of diet) with or without 20% high-amylose maize starch (HAMS) on the thickness of mucus lining the colon in rats. Data are presented as the arithmetic mean ± SEM (n = 8). Values not sharing the same letter are significantly different ($\mathbf{P} < 0.05$).

lead to frank outcomes such as cancer (26–28). Indeed, hereditary non-polyposis colon cancer is associated with a deficiency in capacity for DNA mismatch repair (29). DNA DSBs are potentially more deleterious than SSB in terms of cancer risk as the nature of the repair mechanisms involved may lead to further deleterious changes to the DNA (17,28). In light of this, the present demonstration of a dose-dependent increase in colonic DNA DSB with red meat consumption in the absence of HAMS must at least suggest that such a diet in humans could contribute to increased risk of CRC and other bowel diseases. In support of the potential for high-protein diets to influence risk of CRC through DNA damage events, a recent study of individuals with some common variants of mismatch repair genes associated with CRC found that high intakes of processed meats significantly increased the risk of CRC when individuals had at least one copy of some MSH3 alleles (29).

In this study, cooked white meat was shown to induce DNA damage in a dose-dependent manner, albeit at a level significantly lower than that induced by red meat. There is no evidence to suggest that a high intake of white meat is associated with increased risk of CRC, although increased protein intake *per se* has been linked with greater risk (6). It is known that dietary protein can reach the large bowel and undergo fermentation (30), releasing potentially toxic by-products. To what extent red and white meats differ in their capacity to reach the large bowel and promote fermentation is not known. We propose that the level of DNA damage induced by white meat consumption can be managed effectively by colonocytes but that the greater damage induced by red meat crosses a threshold level above which DNA maintenance mechanisms become ineffective so that genomic stability is compromised. This seems consistent with what is known regarding intracellular responses to stress and with our data. DNA damage results in the activation of a number of pathways, such as the ATM/Chk2 pathway for repair of DNA DSB (31). Many of these pathways are dependent on the tumour suppressor protein p53, which acts to induce cell-cycle arrest at the G_1 checkpoint to enable repair and cell survival (32). In contrast, under conditions where DNA damage is extensive, a greater expression of p53 leads to induction of cell death (33). Also, Deckbar *et al.* (34) have demonstrated that a certain number of DNA DSB may be required to induce a second G_2–M checkpoint. We measured the level of apoptosis in colonocytes in this study and found that red meat increased apoptosis in a dose-dependent manner analogous to the increase in DNA damage. However, apoptosis was unchanged when rats were fed red meat and HAMS, suggesting that any lowering of damage was through less induction of SSB and DSB. In contrast, apoptosis was higher in rats fed white meat plus HAMS than in those fed white meat and the digestible starch. This

Table III. Effects of dietary red meat, white meat and HAMS levels on faecal and caecal SCFA pools in rats

	Dietary treatment												Main effects (*P* values)				
	0% HAMS					20% HAMS							Pooled SEM	HAMS	Protein type	Protein level	Protein type × protein level	Protein type × HAMS	Protein level × HAMS
	Red meat			White meat		Red meat			White meat										
	15%	25%	35%	15%	25%	35%	15%	25%	35%	15%	25%	35%							
Caecal SCFA pools (μM)																			
Acetate	64[a]	105[b]	73[a]	61[a]	68[a]	70[a]	255[c]	216[c]	308[c]	238[c]	217[c]	226[c]	15	<0.0001	<0.05	NS	NS	NS	NS
Propionate	18[a]	25[b]	19[ab]	18[a]	20[ab]	19[a]	95[c]	84[cd]	97[c]	54[e]	71[de]	63[e]	5	<0.0001	<0.001	NS	NS	<0.01	NS
Butyrate	18[a]	30[b]	21[ac]	17[a]	18[a]	22[c]	53[d]	78[e]	43[d]	52[d]	56[de]	45[d]	5	<0.0001	NS	<0.05	NS	NS	NS
Total caecal SCFA	105[a]	170[b]	121[ac]	102[a]	112[ac]	119[c]	412[d]	389[de]	457[d]	355[e]	357[e]	346[e]	19	<0.0001	<0.05	NS	NS	NS	NS
Faecal SCFA pools (μM)																			
Acetate	102[a]	96[ab]	122[a]	86[bc]	88[bc]	74[c]	195[de]	167[d]	197[de]	178[d]	172[d]	256[e]	56	<0.0001	NS	NS	NS	NS	NS
Propionate	21[a]	23[ab]	27[b]	17[c]	18[c]	19[ac]	49[d]	44[d]	55[d]	54[d]	40[d]	54[d]	15	<0.0001	NS	NS	NS	NS	NS
Butyrate	19[ab]	23[ac]	21[ac]	16[b]	19[ab]	19[ab]	24[cd]	33[de]	25[cd]	35[e]	39[ef]	59[f]	13	<0.0001	<0.05	NS	NS	<0.05	NS
Total faecal SCFA	150[a]	152[a]	180[b]	126[c]	133[ac]	121[c]	276[d]	252[d]	286[d]	275[d]	263[d]	386[e]	78	<0.0001	NS	NS	NS	NS	NS

Values are presented as the mean ($n = 8$) and the pooled SEM.
[a–f]Mean values in a row with unlike superscript letters were significantly different ($P < 0.05$).

Table IV. Effects of dietary red meat, white meat and HAMS levels on faecal and caecal phenol and *p*-cresol in rats

	Dietary treatment										Main effects (*P* values)				
	0% HAMS				20% HAMS						Pooled SEM	HAMS	Protein type	Protein level	Protein level × protein level	Protein type × HAMS	Protein type × HAMS
	Red meat		White meat		Red meat		White meat										
	15%	25%	15%	25%	35%	15%	25%	35%	15%	25%	35%						
Caecal (μg/g)																	
Phenol	6.7^{a}	4.8^{ab}	4.1^{b}	2.8^{b}	5.8^{ab}	3.8^{b}	0.4^{cd}	0.4^{cd}	0.5^{c}	0.3^{d}	0.3^{d}	0.5^{c}	0.8	<0.0001	NS	NS	NS	NS	NS
p-cresol	5.5^{ab}	17.9^{c}	20.7^{c}	3.5^{ad}	5.1^{ab}	22.7^{c}	3.3^{ad}	4.6^{ab}	5.9^{b}	3.7^{ad}	1.1^{e}	2.9^{de}	1.5	<0.0001	<0.05	<0.0001	<0.01	NS	<0.0001
Faecal (μg/g)																			
Phenol	13.5^{a}	2.9^{b}	3.5^{bc}	2.7^{c}	4.5^{b}	2.0^{c}	0.3^{de}	0.4^{d}	0.5^{d}	0.2^{e}	0.2^{e}	0.2^{e}	1	<0.001	NS	NS	NS	NS	NS
p-cresol	29.1^{a}	18.6^{a}	17.3^{a}	6.9^{b}	7.9^{b}	18.8^{a}	1.7^{c}	2.0^{c}	1.9^{c}	2.1^{c}	1.1^{c}	1.1^{c}	3	<0.0001	<0.05	<0.0001	NS	<0.05	NS

Values are presented as the mean (n = 8) and the pooled SEM.
[a–e]Mean values in a row with unlike superscript letters were significantly different ($P < 0.05$).

indicates that apoptosis may have contributed more protection when white meat was fed with HAMS.

The epidemiological data on the role of dietary fibre in CRC are somewhat ambiguous. A meta-analysis of a number of prospective studies concluded that fibre did not confer any protection (35). In contrast, a large European multicentre study showed significant dose-dependent risk reduction with increased fibre consumption (36). The discrepancy can be reconciled if the definition of dietary fibre includes RS. There are data showing that non-starch polysaccharides may not be an important contributor to protection as RS in groups at low risk (6). Fermentation of RS in the large bowel results in increased production of SCFA, including butyrate, which is then used by colonocytes for energy and maintenance of a normal phenotype through enhanced apoptotic activity (37,38). Our previous studies have shown that dietary HAMS protects against protein-induced colonic DNA damage in rats through a mechanism that correlates well with levels of butyrate produced in the large bowel (9). Currently, we have confirmed increased SCFA production by 20% dietary HAMS. Now, we have shown that HAMS not only protects against DNA SSB but DSB as well. We have noted earlier that in the absence of dietary HAMS that increasing dietary white meat did not induce an increased apoptotic response whereas red meat did; seeming to suggest that the higher level of damage by red meat crossed a threshold beyond which apoptosis was induced. It is therefore interesting to note that there was an effect of HAMS on apoptosis levels (an increase) when white meat, but not red meat, was consumed. The levels for red meat remained high. This is consistent with RS (or products of RS fermentation) improving the ability of cells to detect DNA breaks and/or lower the threshold at which apoptosis cuts in. DNA damage was measured using the comet assay and only cells that did not appear to be necrotic or undergoing apoptosis were used for analyses. Therefore, the relatively low levels of colonocyte DNA damage observed for HAMS treatments suggest that the non-apoptotic, surviving cells are relatively free of damage, if it is assumed that the levels observed are at baseline (normal).

In an earlier rat study demonstrating protection against red meat-induced DNA damage, HAMS was fed at 48% of the diet, a level far higher than would normally be consumed by humans (8). The present demonstration of protection with 20% HAMS is important as it indicates that inclusion of RS in the diet at levels that could be acceptable to humans could lower risk of colorectal disease. We have shown previously that as little as 10% HAMS (i.e. ∼5% RS) conferred significant protection against protein-induced SSB (10). This translates to an intake of RS which could be achieved by humans (39).

One of the common explanations for the association between increased risk of CRC and red meat consumption is increased formation of heterocyclic amines during cooking. In the present study, both red and white meats were cooked using the same method, eliminating it as a factor in explaining their different effects on colonic DNA damage. However, red meats contain greater amounts of haem in comparison with poultry and haem is known to stimulate production of genotoxic endogenous *N*-nitrosocompounds in the human gut (40,41). Moreover, endogenous *N*-nitrosation can lead to formation of promutagenic and toxic DNA adducts such as O^6-carboxymethyl guanine (42). Also, red meat is known to be high in sulphur amino acids and a human study has shown that red meat consumption dose dependently increases faecal sulphide (43). Carbohydrates are a preferred substrate for many colonic bacteria and in its absence bacteria may also degrade the sulphomucins of the mucus barrier and thereby also generate hydrogen sulphide (44). Increased protein fermentation as a consequence of high-protein diets can also produce harmful compounds such as phenols, cresol, indoles, amines and ammonia (45). High concentrations of these compounds are toxic to the epithelium and may promote genomic instability. A recent rodent study demonstrated that undigested protein promoted azoxymethane-induced adenocarcinoma in the small intestine (46). However, these toxic compounds implicated in bowel cancer (47) are reduced when RS is added to the diet (48), whereas other by-products such as *N*-nitrosocompound may not be affected by RS in humans (49). The present study supports this

by demonstrating a significant reduction in concentrations of phenol and cresol concentrations in caecal content and faeces when HAMS was included in the diet. Accumulation of harmful by-products of protein metabolism may be reduced by the fermentation of carbohydrate. Moreover, lowering of colonic pH through increased SCFA produced in response to RS fermentation may limit the production of ammonia.

Loss of barrier function is a prominent feature of inflammatory bowel diseases and high dietary protein has been related to loss of remission from ulcerative colitis (50). We have shown a significant inverse relationship between colonic DNA damage, especially DSB, and thickness of the colonic mucus layer. Rats consuming 25 or 35% red meat or 25% white meat without RS had a significant reduction in colonic mucus layer thickness an effect which was reversed when HAMS was added to the diet. Previous studies have suggested that SCFA increase colonic mucin secretion *in vivo* (51,52). Moreover, a study conducted using human biopsy samples showed butyrate in particular reduced sulphide-induced mucosal hyperproliferation (53). In addition, a previous study demonstrated that colonic mucus layer thickness was positively related to dietary HAMS levels, and caecal butyrate pools showed a stronger relationship than other SCFA (10). Our data support an important role for dietary RS in maintaining the mucus barrier in the colon as one of the means by which it contributes to large bowel health.

Previous studies have demonstrated that inclusion of 48% HAMS in the diet increased colon and small intestine lengths (8,9). In the present study, there was an increase in colon length, but not small intestine length, when rats were fed the 20% HAMS diets. These findings indicate that RS consumption may stimulate the release of hormones and growth factors to facilitate gut growth and these could also influence the growth of organs at sites distant to the gut.

In conclusion, we have demonstrated that an increase in red meat consumption increases colonic DNA damage and thinning of the colonic mucus layer to a greater extent than white meat in a rat model. We have shown for the first time that dietary red meat increases colonic DNA DSB, a more severe form of damage than SSB. Inclusion of HAMS as a source of RS in the diet attenuated red and white meat-induced DNA damage, restored thickness of the colonic mucus layer, increased levels of apoptosis, increased large bowel SCFA concentrations and reduced harmful by-products of protein metabolism. It must be recognized that in the rat SCFA production is located principally in the caecum while in humans and other omnivorous species (e.g. the pig) fermentation occurs in the proximal colon (54). Nevertheless, the rat is a useful model for initial investigation and our findings support recent epidemiological studies on diet and risk of CRC and suggest that higher levels of genotoxic products formed in the large bowel of individuals consuming red meat versus white meat in the absence of dietary fibre in the form of RS lead to greater loss of genomic stability in the long term.

Acknowledgements

The authors would like to thank Dr Pat Buckley and Dr Mike Nordstrom for helpful discussion and Corinna Bennett and Debbie Davies for technical assistance and advice.

Conflict of Interest Statement: None declared.

References

1. Norat,T. *et al*. (2005) Meat, fish, and colorectal cancer risk: the European prospective investigation into cancer and nutrition. *J. Natl Cancer Inst.*, **97**, 906–916.
2. Chao,A. *et al*. (2005) Meat consumption and risk of colorectal cancer. *JAMA*, **293**, 172–182.
3. Larsson,S.C. *et al*. (2005) Red meat consumption and risk of cancers of the proximal colon, distal colon and rectum: the Swedish mammography cohort. *Int. J. Cancer*, **113**, 829–834.
4. Jain,M. *et al*. (1980) A case-control study of diet and colorectal cancer. *Int. J. Cancer*, **26**, 757–768.
5. English,D.R. *et al*. (2004) Red meat, chicken, and fish consumption and risk of colorectal cancer. *Cancer Epidemiol. Biomark. Prev.*, **13**, 1509–1514.
6. Cassidy,A. *et al*. (1994) Starch intake and colorectal cancer risk: an international comparison. *Br. J. Cancer*, **69**, 937–942.
7. Toden,S. *et al*. (2005) Resistant starch attenuates colonic DNA damage induced by higher dietary protein in rats. *Nutr. Cancer*, **51**, 45–51.
8. Toden,S. *et al*. (2006) Resistant starch prevents colonic DNA damage induced by high dietary cooked red meat or casein in rats. *Cancer Biol. Ther.*, **5**, 267–272.
9. Toden,S. *et al*. (2007) Differential effects of dietary whey, casein and soya on colonic DNA damage and large bowel SCFA in rats fed diets low and high in resistant starch. *Br. J. Nutr.*, **97**, 535–543.
10. Toden,S. *et al*. (2007) Dose-dependent reduction of dietary protein-induced colonocyte DNA damage by resistant starch in rats correlates more highly with caecal butyrate than with other short chain fatty acids. *Cancer Biol. Ther.*, **6**, 253–258.
11. Le Leu,R.K. *et al*. (2003) Effect of resistant starch on genotoxin-induced apoptosis, colonic epithelium, and lumenal contents in rats. *Carcinogenesis*, **24**, 1347–1352.
12. Rieger,M.A. *et al*. (1999) A diet high in fat and meat but low in dietary fibre increases the genotoxic potential of 'faecal water'. *Carcinogenesis*, **20**, 2311–2316.
13. Allinger,U.G. *et al*. (1989) Shift from a mixed to a lactovegetarian diet: influence on acidic lipids in fecal water—a potential risk factor for colon cancer. *Am. J. Clin. Nutr.*, **50**, 992–996.
14. Glinghammar,B. *et al*. (1997) Shift from a dairy product-rich to a dairy product-free diet: influence on cytotoxicity and genotoxicity of fecal water—potential risk factors for colon cancer. *Am. J. Clin. Nutr.*, **66**, 1277–1282.
15. Venturi,M. *et al*. (1997) Genotoxic activity in human faecal water and the role of bile acids: a study using the alkaline comet assay. *Carcinogenesis*, **18**, 2353–2359.
16. Le Leu,R.K. *et al*. (2005) A synbiotic combination of resistant starch and bifidobacterium lactis facilitates apoptotic deletion of carcinogen-damaged cells in rat colon. *J. Nutr.*, **135**, 996–1001.
17. Stark,J.M. *et al*. (2004) Genetic steps of mammalian homologous repair with distinct mutagenic consequences. *Mol. Cell. Biol.*, **24**, 9305–9316.
18. Reeves,P.G. *et al*. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.*, **123**, 1939–1951.
19. Morita,T. *et al*. (2005) Comparative effects of acetylated and unmodified high-amylose maize starch in rats. *Starch*, **57**, 246–253.
20. Olive,P.L. *et al*. (2006) The comet assay: a method to measure DNA damage in individual cells. *Nat. Protoc.*, **1**, 23–29.
21. Helma,C. *et al*. (2000) A public domain image-analysis program for the single-cell gel-electrophoresis (comet) assay. *Mutat. Res.*, **466**, 9–15.
22. Patten,G.S. *et al*. (2002) Dietary fish oil increases acetylcholine- and eicosanoid-induced contractility of isolated rat ileum. *J. Nutr.*, **132**, 2506–2513.
23. Murray,K.E. *et al*. (1988) Determination of simple phenols in faeces and urine by high-performance liquid chromatography. *J. Chromatogr.*, **431**, 143–149.
24. Blocher,D. (1982) DNA double strand breaks in Ehrlich ascites tumour cells at low doses of x-rays. I. Determination of induced breaks by centrifugation at reduced speed. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.*, **42**, 317–328.
25. Bryant,P.E. (1984) Enzymatic restriction of mammalian cell DNA using Pvu II and Bam H1: evidence for the double-strand break origin of chromosomal aberrations. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.*, **46**, 57–65.
26. Friedberg,E.C. (2001) How nucleotide excision repair protects against cancer. *Nat. Rev. Cancer.*, **1**, 22–33.
27. Kastan,M.B. *et al*. (2004) Cell-cycle checkpoints and cancer. *Nature*, **432**, 316–323.
28. Rodrigue,A. *et al*. (2006) Interplay between human DNA repair proteins at a unique double-strand break *in vivo*. *EMBO J.*, **25**, 222–231.
29. Berndt,S.I. *et al*. (2007) Mismatch repair polymorphisms and the risk of colorectal cancer. *Int. J. Cancer*, **120**, 1548–1554.
30. Morita,T. *et al*. (1998) Resistant proteins alter cecal short-chain fatty acid profiles in rats fed high amylose cornstarch. *J. Nutr.*, **128**, 1156–1164.
31. Zhou,B.B. *et al*. (2000) The DNA damage response: putting checkpoints in perspective. *Nature*, **408**, 433–439.
32. Harris,S.L. *et al*. (2005) The p53 pathway: positive and negative feedback loops. *Oncogene*, **24**, 2899–2908.
33. Vousden,K.H. *et al*. (2007) p53 in health and disease. *Nat. Rev. Mol. Cell Biol.*, **8**, 275–283.

34. Deckbar,D. *et al*. (2007) Chromosome breakage after G2 checkpoint release. *J. Cell Biol.*, **176**, 749–755.
35. Fuchs,C.S. *et al*. (1999) Dietary fiber and the risk of colorectal cancer and adenoma in women. *N. Engl. J. Med.*, **340**, 169–176.
36. Bingham,S.A. *et al*. (2003) Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. *Lancet*, **361**, 1496–1501.
37. Thompson,C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science*, **267**, 1456–1462.
38. Singh,B. *et al*. (1997) Butyrate can act as a stimulator of growth or inducer of apoptosis in human colonic epithelial cell lines depending on the presence of alternative energy sources. *Carcinogenesis*, **18**, 1265–1270.
39. Birkett,A.M. *et al*. (1997) Dietary intake and faecal excretion of carbohydrate by Australians: importance of achieving stool weights greater than 150 g to improve faecal markers relevant to colon cancer risk. *Eur. J. Clin. Nutr.*, **51**, 625–632.
40. Bingham,S.A. *et al*. (1996) Does increased endogenous formation of N-nitroso compounds in the human colon explain the association between red meat and colon cancer? *Carcinogenesis*, **17**, 515–523.
41. Cross,A.J. *et al*. (2003) Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. *Cancer Res.*, **63**, 2358–2360.
42. Shuker,D.E. *et al*. (1997) Nitrosated glycine derivatives as a potential source of O6-methylguanine in DNA. *Cancer Res.*, **57**, 366–369.
43. Magee,E.A. *et al*. (2000) Contribution of dietary protein to sulfide production in the large intestine: an *in vitro* and a controlled feeding study in humans. *Am. J. Clin. Nutr.*, **72**, 1488–1494.
44. McGarr,S.E. *et al*. (2005) Diet, anaerobic bacterial metabolism, and colon cancer: a review of the literature. *J. Clin. Gastroenterol.*, **39**, 98–109.
45. Visek,W.J. (1978) Diet and cell growth modulation by ammonia. *Am. J. Clin. Nutr.*, **31**, S216–S220.
46. Le Leu,R.K. *et al*. (2007) Effect of dietary resistant starch and protein on colonic fermentation and intestinal tumourigenesis in rats. *Carcinogenesis*, **28**, 240–245.
47. Bone,E. *et al*. (1976) The production of urinary phenols by gut bacteria and their possible role in the causation of large bowel cancer. *Am. J. Clin. Nutr.*, **29**, 1448–1454.
48. Govers,M.J. *et al*. (1999) Wheat bran affects the site of fermentation of resistant starch and luminal indexes related to colon cancer risk: a study in pigs. *Gut*, **45**, 840–847.
49. Silvester,K.R. *et al*. (1997) Effect of meat and resistant starch on fecal excretion of apparent *N*-nitroso compounds and ammonia from the human large bowel. *Nutr. Cancer*, **29**, 13–23.
50. Jowett,S.L. *et al*. (2004) Influence of dietary factors on the clinical course of ulcerative colitis: a prospective cohort study. *Gut*, **53**, 1479–1484.
51. Shimotoyodome,A. *et al*. (2000) Short chain fatty acids but not lactate or succinate stimulate mucus release in the rat colon. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.*, **125**, 525–531.
52. Cummings,J.H. *et al*. (1991) The control and consequences of bacterial fermentation in the human colon. *J. Appl. Bacteriol.*, **70**, 443–459.
53. Christl,S.U. *et al*. (1996) Antagonistic effects of sulfide and butyrate on proliferation of colonic mucosa: a potential role for these agents in the pathogenesis of ulcerative colitis. *Dig. Dis. Sci.*, **41**, 2477–2481.
54. Topping,D.L. *et al*. (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol. Rev.*, **81**, 1031–1064.

Received July 18, 2007; revised September 13, 2007; accepted September 14, 2007