

## 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

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Feeding higher levels of dietary animal protein (as casein or red meat) increases colonic DNA damage and thins the colonic mucus barrier in rats. Feeding resistant starch (RS) reverses these changes and increases large bowel SCFA. The present study examined whether high dietary dairy (casein or whey) or plant (soya) proteins had similar adverse effects and whether dietary RS was protective. Adult male rats were fed diets containing 15 or 25 % casein, whey or soya protein with or without 48 % high amylose starch (as a source of RS) for 4 weeks. DNA damage was measured in isolated colonocytes using the comet assay. Higher dietary casein and soya (but not whey) increased colonocyte DNA damage. DNA damage was highest with soya when fed at 15 or 25 % protein without RS. Dietary RS attenuated protein-induced colonocyte DNA damage in all groups but it remained significantly higher in rats fed 25 % soya compared with those fed 15 % protein. Dietary protein level did not affect colonic mucus thickness overall but the barrier was thinner in rats fed high dietary casein. This effect was reversed by feeding RS. Caecal total SCFA and butyrate pools were higher in rats fed RS compared with digestible starch. Caecal and faecal SCFA were unrelated to genetic damage but correlated with mucus thickness. The present data confirm that higher dietary protein affected colonocyte DNA and colonic mucus thickness adversely but that proteins differ in their effects on these indices of colon health. The data show also that these changes were reversed by RS.

### Casein: Whey protein: Soya protein: Resistant starch: Colonic genotoxicity

Diet is implicated strongly in the pathogenesis of serious large bowel diseases. Recent large population studies have found that high intakes of red and/or processed meat are positive risk factors of colo-rectal cancer (Cassidy *et al.* 1994; Rieger *et al.* 1999; Norat *et al.* 2002, 2005; Chao *et al.* 2005; Larsson *et al.* 2005). The components of these meat-based foods responsible for increased risk remain to be established. It has been suggested that minor meat components such as haem iron contribute to this increased risk. In a meta-analysis of prospective population studies, Cassidy *et al.* (1994) identified dietary protein *per se* as an independent risk factor for colo-rectal cancer. High protein intakes have other adverse effects on bowel health as evidenced by an increased likelihood of relapse for ulcerative colitis patients (Jowett *et al.* 2004). Inflammatory bowel disease is a risk factor for large bowel cancer (Chen *et al.* 2005), supporting a role for the protein component in disease aetiology.

We have shown previously that high dietary levels of animal-derived proteins (casein and red meat) increased colonocyte DNA damage in rats (Toden *et al.* 2005, 2006). Genomic damage is a prerequisite for cancer and the data could help to explain the associations between the consumption of red meat and meat products and large bowel cancer (Norat *et al.* 2005). Both protein types also induced a thinning of the mucus barrier in the colon which could be a potential contributory factor in inflammatory bowel disease

(Shaoul *et al.* 2004). However, the fact that high dietary levels of casein had the same effects as red meat suggest that the effect was not specific to one type of protein. Our knowledge of the role of dietary proteins in cancer aetiology in general is poor and it remains to be established whether other protein types have similar effects. For example, whey protein consumption is generally regarded as being protective against experimental colo-rectal cancer (Belobrajdic *et al.* 2003; Xiao *et al.* 2005) and also can reduce aberrant crypt foci compared with other protein types (McIntosh & Le Leu, 2001; Belobrajdic *et al.* 2003). However, it is not known whether its actions are affected by dietary inclusion level. The situation with soya is confused with several contradictory experimental studies on colon cancer risk and soya protein consumption (Govers *et al.* 1993; McIntosh *et al.* 1995; Hakkak *et al.* 2001b; Badger *et al.* 2005). Isoflavones and other phytochemicals are often present in soya-based products and have been shown to inhibit cancer cell growth and induce apoptosis (Hakkak *et al.* 2001b). However, several rodent studies showed that soyabean protein increased colo-rectal cancer risk, induced damage to the colonic epithelium and stimulated colonocyte proliferation when compared with a casein diet (Govers *et al.* 1993; McIntosh *et al.* 1995). Soya protein isolates have been investigated extensively also for their ability to lower plasma cholesterol relative to casein (Lin *et al.* 2004), again suggesting some differences in nutritional properties.

**Abbreviations:** RS, resistant starch.

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Intake of fruits, vegetables and grains is linked to reduction of colo-rectal cancer risk (Potter *et al.* 1993; Giovannucci & Willett, 1994; Steinmetz & Potter, 1996). These foods contain non-starch polysaccharides (NSP; major components of dietary fibre) which are thought to contribute to their protective action. Many also contain resistant starch (RS) which can also contribute to their protective effect (Topping & Clifton, 2001). RS is that fraction of starch which enters the large bowel undigested (Englyst *et al.* 1982) and increases colonic fermentation and large bowel and faecal SCFA concentrations (Cummings & Macfarlane, 1991). Butyrate, a major SCFA, is the preferred energy source of colonocytes (Clausen & Mortensen, 1995; Ritzhaupt *et al.* 1998) and appears to provide protection against activities associated with carcinogenesis (Lupton, 1995). Furthermore, epidemiological studies have related a reduced risk of colon cancer to increased consumption of starch (Cassidy *et al.* 1994; Bingham *et al.* 2003). Our previous studies (Toden *et al.* 2005, 2006) also showed that dietary RS protected against casein and red meat-induced colonic DNA damage and some loss of the mucosal barrier. The previous findings provide support for a role of RS in maintaining colonic integrity and lowering the risk of serious large bowel disease. However, not all experimental studies support the protective effects of RS. One study reported that lipid peroxidation-induced DNA damage was increased in colonic mucosa of health subjects when they were fed the high RS diets (Wacker *et al.* 2002). Another study showed that RS increased intestinal tumour formation of Apc1638N mouse model compared with those fed the control diets (Williamson *et al.* 1999).

The question of the differential effects of animal and plant proteins is an important one and remains open. In the present study, we have sought to examine the effects of three different types of dietary proteins, casein, whey and soya, on colonic DNA damage, colonic mucus barrier thickness and SCFA levels in the caecum and faeces of rats. In addition, we have examined the ability of dietary RS to modulate the effects of changes in the colon induced by these proteins.

## Materials and methods

### Animals and diets

Adult male Sprague-Dawley rats (*n* 96) weighing approximately 200 g were obtained from the Animal Resource Centre, Murdoch University, Perth, Western Australia. Rats were housed in wire-bottomed cages in a room with controlled heating and lighting (20–23°C with a 12 h light–dark cycle) and had free access to food and water. They were allocated randomly to twelve groups (eight per group) and fed one of twelve diets (Table 1) for 4 weeks. Each cage had a ceramic bowl to ensure that the animals had access to a surface other than wire mesh.

The diets, which were based on the AIN-93G diet formulation, contained 15 or 25 % casein (New Zealand Milk, Auckland, New Zealand), whey isolate (Balance Muscle Technologies, Auckland, New Zealand) and soya isolate (Phyto Foods Australia Pty Ltd, NSW, Australia), with or without 48 % high amylose maize starch (Hi-maize™; National Starch and Chemical Company, Sydney, NSW, Australia) as a source of RS. Diets without high amylose starch contained highly digestible starch

**Table 1.** Composition of experimental diets (g/kg diet)\*

	Dietary treatment											
	0% High amylose maize starch						48% High amylose maize starch					
	15% Casein	25% Casein	15% Whey	25% Whey	15% Soya	25% Soya	15% Casein	25% Casein	15% Whey	25% Whey	15% Soya	25% Soya
Casein	150	250	0	0	0	0	150	250	0	0	0	0
Whey	0	0	150	250	0	0	0	0	150	250	0	0
Soya	0	0	0	0	150	250	0	0	0	0	150	250
Maize starch	580	480	580	480	580	480	100	480	100	480	100	480
High amylose maize starch	0	0	0	0	0	0	480	480	480	480	480	480
Sucrose	100	100	100	100	100	100	100	100	100	100	100	100
Sunflower oil	70	70	70	70	70	70	70	70	70	70	70	70
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	35	35	35	35	35	35	35	35	35	35	35	35
terf-Butyl hydroquinol	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014

\*Diets based on AIN-93 (G) formulation.

(Cornstarch 3401C; National Starch and Chemical Company). All diets contained 5% wheat bran as the fibre source.

All procedures involving animals were approved by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Health Sciences and Nutrition Animal Ethics Committee and the University of Adelaide Animal Ethics Committee and conformed to published guidelines (National Health and Medical Research Council, 2004).

#### *Sampling procedures*

In the final 4 d of the experiment rats were placed in metabolic cages to collect faecal output. At the conclusion of the study the rats were anaesthetized with 4% halothane/oxygen and gut tissues and digesta were collected as described previously (Toden *et al.* 2005). Faecal and caecal digesta samples were frozen for subsequent analyses.

#### *Comet assay*

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. These cells were used for the measurement of DNA strand breaks using the single-cell gel electrophoresis (comet) assay as described previously (Anderson *et al.* 1999). Comet tail moment is the product of tail length and the fraction of DNA in the tail and was calculated for fifty cells from each of three slides per rat. Tail moment was calculated by Scion Image Beta 4.02 image processing and analysis software (Scion Corp., Frederick, MD, USA) using a public domain macro (Helma & Uhl, 2000).

#### *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 program as described previously (Toden *et al.* 2005). For each animal, ten measurements were taken at different points along four tissue segments to give forty thickness measurements in total.

#### *SCFA*

Faecal and caecal contents were thawed then distilled and homogenised with 20  $\mu$ l 1.68 mM-heptanoic acid (internal standard). 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 Zebtron ZB-FFAP column (0.53 mm  $\times$  30 m) (Phenomenex, Torrance, CA, USA) as described previously (Patten *et al.* 2002).

#### *Statistical analyses*

Data are presented as means and their standard errors for each treatment group except in tables (where they are presented

with pooled standard error values). The effect of RS and protein, and their interactions, were determined by three-way ANOVA and differences between treatments were analysed *post hoc* by Tukey's test. The relationship between caecal and faecal variables, colonic DNA damage and mucus layer thickness was determined by regression analysis. Data underwent logarithmic transformation when needed. Analyses were performed using a Statistical Analysis Systems statistical software program (version 8.02: SAS Institute, Cary, NC, USA). A value of  $P < 0.05$  was used as the criterion of significance.

## **Results**

### *Body weight gain and daily food and water intakes*

The mean initial live weight for all groups combined was 241 (SEM 2) g. Neither live weight gain (data not shown) nor final body weight differed between the experimental diets (mean final weight 353 (SEM 4) g) over the 4-week study. There was no effect of protein type, protein level or RS on the daily food intake of the animals (Table 2). Neither RS nor protein type affected water intake but there was an independent effect of protein type and an interactive effect of protein level and type on daily water intake (both  $P < 0.05$ ). Water consumption was highest in rats fed the 25% soya diet.

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

The wet weight of caecal content in RS-fed rats was 5.1 times higher than those fed standard starch ( $P < 0.001$ ; Table 2). There was also a significant interaction between protein type and RS. Caecal tissue weight was unaffected by protein type or level but was significantly greater in rats fed RS (Table 2). There was also an interaction between dietary protein type and RS with a significantly lower weight in rats fed 25% whey and RS compared with the other groups fed RS. Caecal digesta pH values were greater than 7 in all rats fed the standard starch diets with the exception of those fed the 25% soya diet (Table 2). There was a small effect of protein type (but not level) on pH and a significant interaction between protein type and level and between these effects and RS.

Faecal output was unaffected by dietary protein level but was significantly higher in all groups of rats fed RS (Table 2). The type of dietary protein interacted with RS with a significantly lower output of faeces in rats fed 25% casein with RS compared with other groups fed the high RS diet. The pH of faeces followed the same trend as the caecal values (i.e.  $> 7$ ) in rats fed the low RS diet with significantly greater acidity in rats fed RS. There were effects of protein level and type with the highest pH (close to 8) in rats fed whey and standard starch.

Daily urine output was unaffected by RS (Table 2) but was affected significantly by protein level with higher outputs in all groups fed 25% protein.

There were increases in both weight and length of the colon and small intestine when rats were fed the RS compared to those on diets without RS (all  $P < 0.001$ ; Table 3). However, protein type did not influence weight or length of either the colon or small intestine independently, but did interact significantly with RS.

**Table 2.** Effects of dietary casein, whey, soya and high amylose starch maize (resistant starch, RS) levels on daily food and water intakes, faecal and urinary outputs, and caecal and faecal measurements in rats\*(Mean values with their the pooled standard error, *n* 8)

	Dietary treatment												Main effects ( <i>P</i> values)							
	0 % High amylose maize starch						48 % High amylose maize starch						Pooled SEM	RS level	Protein type	Protein level	Protein type × Protein level	Protein type × RS	Protein level × RS	All
	15% Casein	25% Casein	15% Whey	25% Whey	15% Soya	25% Soya	15% Casein	25% Casein	15% Whey	25% Whey	15% Soya	25% Soya								
Food intake (g/d)	17.2 <sup>a</sup>	18.7 <sup>ab</sup>	17.9 <sup>ab</sup>	17.3 <sup>a</sup>	18.1 <sup>ab</sup>	17.2 <sup>a</sup>	19.1 <sup>b</sup>	17.1 <sup>a</sup>	19.3 <sup>b</sup>	17.8 <sup>ab</sup>	17.4 <sup>b</sup>	17.8 <sup>ab</sup>	0.6	0.32	0.54	0.14	0.48	0.52	0.15	<0.05
Water intake (ml/d)	18.0 <sup>a</sup>	23.0 <sup>a</sup>	20.0 <sup>a</sup>	24.2 <sup>a</sup>	21.3 <sup>a</sup>	24.5 <sup>a</sup>	22.6 <sup>a</sup>	24.3 <sup>a</sup>	22.8 <sup>a</sup>	26.8 <sup>b</sup>	20.7 <sup>a</sup>	35.7 <sup>c</sup>	1.8	0.06	0.29	<0.05	<0.05	0.26	0.46	0.23
Caecal measurements																				
Contents weight (g)	0.8 <sup>a</sup>	1.0 <sup>a</sup>	0.9 <sup>a</sup>	1.3 <sup>a</sup>	1.1 <sup>a</sup>	2.0 <sup>a</sup>	5.7 <sup>b</sup>	5.6 <sup>b</sup>	6.9 <sup>b</sup>	5.5 <sup>b</sup>	6.1 <sup>b</sup>	5.6 <sup>b</sup>	0.4	<0.001	0.36	0.63	0.4	<0.001	0.47	0.46
Tissue weight (g)	1.0 <sup>a</sup>	1.2 <sup>a</sup>	1.2 <sup>a</sup>	1.1 <sup>a</sup>	1.1 <sup>a</sup>	1.5 <sup>a</sup>	3.9 <sup>bc</sup>	4.1 <sup>c</sup>	3.7 <sup>bc</sup>	2.8 <sup>d</sup>	3.0 <sup>bd</sup>	3.5 <sup>bc</sup>	0.2	<0.001	0.34	0.88	0.3	<0.001	0.58	0.50
pH	7.77 <sup>a</sup>	7.57 <sup>a</sup>	7.84 <sup>a</sup>	7.89 <sup>a</sup>	7.31 <sup>a</sup>	6.56 <sup>c</sup>	6.03 <sup>bc</sup>	5.68 <sup>b</sup>	5.84 <sup>b</sup>	5.83 <sup>b</sup>	6.16 <sup>bc</sup>	5.60 <sup>b</sup>	0.29	<0.001	<0.05	0.96	<0.001	<0.001	0.53	0.76
Faecal measurements																				
Output (g/d)	1.9 <sup>a</sup>	1.9 <sup>a</sup>	2.4 <sup>a</sup>	2.3 <sup>a</sup>	2.3 <sup>a</sup>	2.4 <sup>a</sup>	4.8 <sup>bc</sup>	3.7 <sup>d</sup>	5.1 <sup>b</sup>	4.6 <sup>c</sup>	4.9 <sup>bc</sup>	5.6 <sup>b</sup>	0.2	<0.001	<0.05	0.32	<0.05	<0.001	0.33	0.11
pH	7.31 <sup>a</sup>	7.22 <sup>a</sup>	7.91 <sup>c</sup>	8.09 <sup>c</sup>	7.02 <sup>a</sup>	7.24 <sup>a</sup>	5.60 <sup>b</sup>	5.81 <sup>b</sup>	5.75 <sup>b</sup>	5.56 <sup>b</sup>	5.83 <sup>b</sup>	5.67 <sup>b</sup>	0.40	<0.001	<0.001	<0.05	0.03	<0.001	0.16	0.60
Urine output (ml/d)	8.3 <sup>a</sup>	12.0 <sup>b</sup>	9.4 <sup>ab</sup>	11.4 <sup>b</sup>	10.0 <sup>a</sup>	12.4 <sup>b</sup>	8.6 <sup>a</sup>	12.2 <sup>b</sup>	9.3 <sup>ab</sup>	13.5 <sup>d</sup>	9.0 <sup>ab</sup>	13.7 <sup>d</sup>	0.90	0.42	0.26	<0.001	<0.001	0.78	0.16	0.73

<sup>a-e</sup> Mean values within a row with unlike superscript letters were significantly different (*P*<0.05).\* For details of procedures, see p. 537. Data were analysed by three-way ANOVA and *post hoc* analysis by Tukey's test.**Table 3.** Effects of dietary casein, whey, soya and high amylose starch maize (resistant starch, RS) levels on length and weight of the colon and small intestine and weight of colonic contents in rats\*(Mean values with their pooled standard error, *n* 8)

	Dietary treatment												Main effects ( <i>P</i> values)							
	0 % High amylose maize starch						48 % High amylose maize starch						Pooled SEM	RS level	Protein type	Protein level	Protein type × Protein level	Protein type × RS	Protein level × RS	All
	15% Casein	25% Casein	15% Whey	25% Whey	15% Soya	25% Soya	15% Casein	25% Casein	15% Whey	25% Whey	15% Soya	25% Soya								
Colon																				
Length (cm)	18.4 <sup>abc</sup>	17.4 <sup>acd</sup>	16.6 <sup>bcd</sup>	16.1 <sup>d</sup>	17.5 <sup>abcd</sup>	16.4 <sup>cd</sup>	18.3 <sup>abc</sup>	18.8 <sup>a</sup>	18.1 <sup>a</sup>	18.5 <sup>ab</sup>	18.6 <sup>a</sup>	18.3 <sup>abc</sup>	0.7	<0.001	0.21	0.40	0.51	<0.05	0.22	0.92
Weight (g)	1.08 <sup>ab</sup>	1.11 <sup>abcd</sup>	1.04 <sup>b</sup>	0.98 <sup>b</sup>	1.03 <sup>b</sup>	1.04 <sup>ab</sup>	1.35 <sup>d</sup>	1.34 <sup>c</sup>	1.19 <sup>abcd</sup>	1.28 <sup>acd</sup>	1.41 <sup>d</sup>	1.39 <sup>d</sup>	0.08	<0.001	0.17	0.88	0.63	<0.001	0.81	0.70
Content (g)	0.55 <sup>abc</sup>	0.50 <sup>ac</sup>	0.27 <sup>c</sup>	0.35 <sup>c</sup>	0.36 <sup>c</sup>	0.80 <sup>abd</sup>	1.01 <sup>d</sup>	0.86 <sup>abd</sup>	0.91 <sup>abd</sup>	0.94 <sup>bd</sup>	1.10 <sup>d</sup>	0.88 <sup>abd</sup>	0.10	<0.001	0.25	0.73	0.53	<0.001	0.12	0.29
Small intestine																				
Weight (g)	7.3 <sup>a</sup>	8.1 <sup>ad</sup>	8.0 <sup>ad</sup>	8.0 <sup>ad</sup>	7.7 <sup>a</sup>	7.7 <sup>a</sup>	9.7 <sup>bc</sup>	10.0 <sup>b</sup>	9.7 <sup>bc</sup>	9.2 <sup>bc</sup>	8.8 <sup>cd</sup>	10.1 <sup>b</sup>	0.3	<0.001	0.71	0.10	0.17	<0.001	0.79	0.10
Length (cm)	106.4 <sup>a</sup>	104.6 <sup>a</sup>	102.5 <sup>a</sup>	105.3 <sup>a</sup>	107.3 <sup>a</sup>	108.4 <sup>abc</sup>	112.0 <sup>bc</sup>	110.5 <sup>bc</sup>	110.6 <sup>bc</sup>	108.8 <sup>bc</sup>	110.9 <sup>bc</sup>	115.9 <sup>b</sup>	2.6	<0.001	0.16	0.67	0.42	<0.05	0.96	0.58

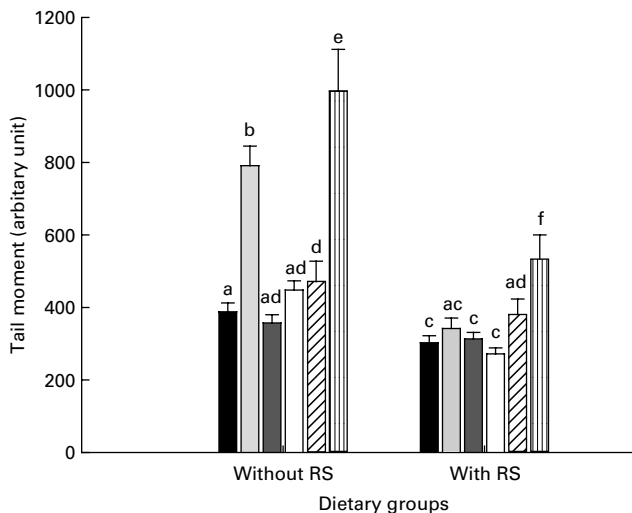
<sup>a-d</sup> Mean values within a row with unlike superscript letters were significantly different (*P*<0.05).\* For details of procedures, see p. 537. Data were analysed by three-way ANOVA and *post hoc* analysis by Tukey's test.

### DNA damage

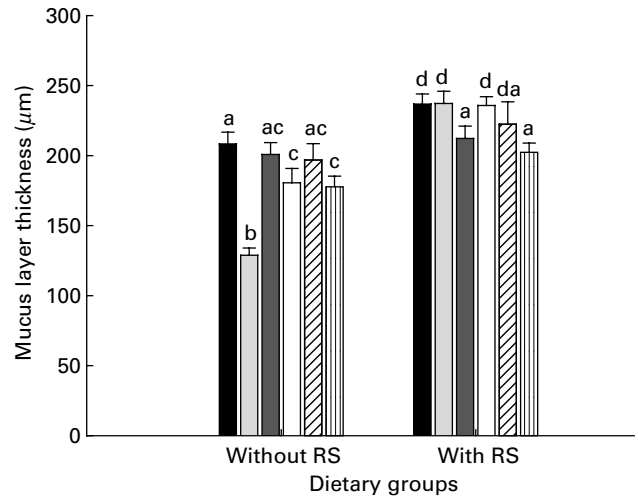
The viability of colonocytes isolated for the comet assay was 92.2 (SEM 2.4) % (all groups combined) and did not differ significantly between groups. When RS was absent from the diet, comet tail moments of isolated colonocytes were approximately twice as great for 25 % casein diets compared with those for 15 % casein diets ( $P < 0.001$ ; Fig. 1). Similarly, the 25 % soya diet increased the tail moment by 87 % compared to the 15 % soya diet ( $P < 0.05$ ). However, there was no significant difference between 15 and 25 % whey diets which had tail moments similar to the 15 % casein and soya groups. Overall, rats fed whey protein showed less colonic DNA damage compared to those fed casein or soya ( $P < 0.001$ ). Inclusion of RS in the diet lowered the level of colonic DNA damage for all three dietary groups ( $P < 0.001$ ). Although RS reduced soya-induced colonic DNA damage, colonocyte comet tail moment of rats fed 25 % soya with RS remained significantly greater than those fed the 15 % soya diet.

### Colonic mucus layer thickness

The colonic mucus layer was thicker in rats fed RS compared to those fed standard maize starch ( $P < 0.001$ ). The 25 % casein diet reduced colonic mucus layer thickness by 62 % compared to the 15 % casein diet in rats consuming low RS ( $P < 0.001$ ), but there was no similar reduction of mucus thickness in rats fed the high soya and whey diets with low RS (Fig. 2). The inclusion of RS in the diet prevented the loss of mucus thickness induced by high casein. Regression analysis indicates that there is a significant inverse relationship



**Fig. 1.** Effects of dietary casein (■, 15%; ■, 25%), whey (▒, 15%; □, 25%), soya (▨, 15%; ▩, 25%) and resistant starch (RS) levels on colonic genetic damage in rats. The comet assay was performed on colonocytes extracted from the colon and the resulting comet tail moments (comet tail length × % DNA in the tail) are presented. For details of procedures, see p. 537. Values are means with their standard errors depicted by vertical bars ( $n = 8$ ). <sup>a–f</sup> Mean values with unlike superscript letters were significantly different ( $P < 0.05$ ). Significant main effects of the dietary groups are RS level ( $P < 0.001$ ), protein type ( $P < 0.001$ ), protein level ( $P < 0.001$ ), protein type × protein level ( $P < 0.01$ ) and protein level × RS ( $P < 0.001$ ).



**Fig. 2.** Effects of dietary casein (■, 15%; ■, 25%), whey (▒, 15%; □, 25%), soya (▨, 15%; ▩, 25%) and resistant starch levels on the thickness of the mucus lining the colon. For details of procedures, see p. 537. Values are means with their standard errors depicted by vertical bars ( $n = 8$ ). <sup>a–d</sup> Mean values with unlike superscript letters were significantly different ( $P < 0.05$ ). Significant main effects of the dietary groups are RS level ( $P < 0.001$ ), protein type × RS ( $P < 0.01$ ) and protein level × RS ( $P < 0.05$ ).

between comet tail moment of colonocytes and mucus thickness ( $n = 96$ ,  $r = -0.41$ ,  $P = 0.001$ ).

### Caecal and faecal SCFA

Caecal and faecal acetate, butyrate, propionate and total SCFA pools were all increased by dietary RS (Table 4). Protein type also affected both caecal and faecal acetate, propionate, butyrate and total SCFA pools (Table 4) and rats fed RS showed significantly lower caecal and faecal pH ( $P < 0.001$ ; Table 2). There was a significant correlation between caecal and faecal pH with respective total SCFA pools (caecal  $r = -0.81$ ,  $P < 0.0001$ ; faecal  $r = -0.67$ ,  $P < 0.0001$ ). DNA damage as measured by the comet assay was found to be significantly associated with several caecal and faecal parameters. The significant relationships seen with DNA damage were with acetate pools (caecal  $r = -0.33$ ,  $P < 0.05$ ; faecal  $r = -0.35$ ,  $P < 0.05$ ), butyrate pools (faecal  $r = 0.34$ ,  $P < 0.05$ ) and total SCFA pools (caecal  $r = -0.402$ ,  $P < 0.001$ ; faecal  $r = -0.36$ ,  $P < 0.05$ ). In addition, a significant relationship was seen with mucus layer thickness and total SCFA pools (caecal  $r = 0.39$ ,  $P < 0.001$ ; faecal  $r = 0.40$ ,  $P < 0.001$ ).

### Discussion

The present data confirm and extend our previous investigations on the effects of dietary protein level and RS on the colonic environment in rats. Specifically, we have shown again that increasing levels of dietary casein from 15% to 25% induces greater DNA strand breakage and thinning of the mucus layer. Up until now, we have only studied proteins of animal origin but these experiments show that a plant protein concentrate (soya) caused even greater colonocyte DNA damage than casein. Collectively, the data suggest that the basic effect was due to the protein content of the diet, rather than minor components such as haem (in meat) or

**Table 4.** Effects of dietary casein, whey, soya and high amylose starch maize (resistant starch, RS) levels on faecal and caecal SCFA pools in rats\* (Mean values with their pooled standard error, *n* 8)

	Dietary treatment												Main effects ( <i>P</i> values)					
	0 % High amylose maize starch						48 % High amylose maize starch						Protein type × Protein level		Protein type × RS level		Protein level × RS level	
	15 % Casein	25 % Casein	15 % Soya	25 % Soya	15 % Whey	25 % Whey	15 % Casein	25 % Casein	15 % Whey	25 % Whey	15 % Soya	25 % Soya	15 % Protein type	25 % Protein level	15 % Protein type × RS level	25 % Protein level × RS level	All	
<b>Caecal SCFA pools (μmol)</b>																		
Acetate	52 <sup>a</sup>	57 <sup>a</sup>	78 <sup>a</sup>	148 <sup>a</sup>	71 <sup>a</sup>	120 <sup>a</sup>	459 <sup>b</sup>	584 <sup>bc</sup>	684 <sup>cd</sup>	903 <sup>e</sup>	575 <sup>bc</sup>	773 <sup>de</sup>	<0.001	<0.05	<0.001	<0.001	<0.05	0.95
Propionate	13 <sup>a</sup>	15 <sup>a</sup>	17 <sup>a</sup>	38 <sup>a</sup>	18 <sup>a</sup>	29 <sup>a</sup>	113 <sup>b</sup>	114 <sup>b</sup>	224 <sup>c</sup>	161 <sup>b</sup>	125 <sup>b</sup>	114 <sup>b</sup>	<0.001	0.54	<0.001	<0.001	0.08	0.32
Butyrate	17 <sup>a</sup>	22 <sup>ad</sup>	25 <sup>ad</sup>	50 <sup>de</sup>	16 <sup>a</sup>	22 <sup>ad</sup>	61 <sup>bc</sup>	47 <sup>bde</sup>	130 <sup>f</sup>	98 <sup>g</sup>	86 <sup>cg</sup>	126 <sup>f</sup>	<0.001	0.43	<0.001	<0.001	0.2	0.12
Total caecal SCFA	86 <sup>a</sup>	98 <sup>a</sup>	125 <sup>a</sup>	248 <sup>a</sup>	110 <sup>a</sup>	178 <sup>a</sup>	642 <sup>b</sup>	749 <sup>b</sup>	1055 <sup>c</sup>	1166 <sup>c</sup>	798 <sup>b</sup>	1020 <sup>c</sup>	<0.001	<0.05	<0.001	<0.001	<0.05	0.48
<b>Faecal SCFA pools (μmol)</b>																		
Acetate	112 <sup>a</sup>	97 <sup>a</sup>	238 <sup>c</sup>	238 <sup>c</sup>	226 <sup>c</sup>	224 <sup>c</sup>	466 <sup>b</sup>	507 <sup>bf</sup>	911 <sup>d</sup>	657 <sup>e</sup>	630 <sup>ef</sup>	721 <sup>e</sup>	<0.001	0.6	<0.001	<0.001	0.45	0.06
Propionate	10 <sup>a</sup>	9 <sup>a</sup>	23 <sup>a</sup>	23 <sup>a</sup>	29 <sup>a</sup>	25 <sup>a</sup>	51 <sup>b</sup>	52 <sup>b</sup>	88 <sup>c</sup>	67 <sup>bc</sup>	62 <sup>b</sup>	61 <sup>b</sup>	<0.001	0.4	<0.001	<0.001	0.44	0.7
Butyrate	2 <sup>a</sup>	2 <sup>a</sup>	14 <sup>ab</sup>	14 <sup>ab</sup>	11 <sup>ab</sup>	10 <sup>ab</sup>	17 <sup>b</sup>	15 <sup>b</sup>	53 <sup>d</sup>	41 <sup>cd</sup>	40 <sup>cd</sup>	35 <sup>c</sup>	<0.001	0.41	<0.001	<0.001	0.23	0.74
Total SCFA	130 <sup>ab</sup>	113 <sup>c</sup>	289 <sup>d</sup>	289 <sup>d</sup>	277 <sup>bd</sup>	269 <sup>abd</sup>	541 <sup>c</sup>	581 <sup>c</sup>	1061 <sup>e</sup>	773 <sup>f</sup>	742 <sup>e</sup>	828 <sup>f</sup>	<0.001	0.51	<0.001	<0.001	0.34	0.66

<sup>a–g</sup> Mean values within a row with unlike superscript letters were significantly different (*P* < 0.05).

\* For details of procedures, see p. 537. Data were analysed by three-way ANOVA and *post hoc* analysis by Tukey's test.

phytochemicals (in soya). However, this does not explain the lack of a genetic damaging effect of higher dietary whey protein. A recent study showed that dietary exposure to both whey and soya proteins altered colonic global gene expression profiles, including repression of a key regulatory gene for cell cycle progression in rats with azoxymethane-induced colon tumours (Xiao *et al.* 2005). In addition, previous rodent studies, using colon tumour induction with azoxymethane or 1,2-dimethylhydrazine, showed that whey protein was protective compared to casein, red meat and soyabean diets (Hakkak *et al.* 2001a; McIntosh & Le Leu, 2001; Belobrajdic *et al.* 2003). Another study demonstrated that bovine lactoferrin, a major whey component, reduced colon carcinogenesis in the post-initiation stage in azoxymethane-treated rats, with significant reduction of adenocarcinoma incidence (Tsuda *et al.* 2000). A suggested protective mechanism of whey is its capacity to increase tissue glutathione. Whey protein has been shown to raise hepatic and (to a lesser extent) colonic glutathione concentrations in rats (McIntosh *et al.* 1995) which may facilitate deactivation of xenobiotics via glutathione transferase activities (Bounous, 2000). Animal studies have shown that a fraction of dietary protein (resistant protein) escapes into the large bowel in a manner analogous to RS (Morita *et al.* 1998). A loss of protein from the small intestine has been reported in human subjects with ileostomy (McBurney *et al.* 1988). However, there seems to be no difference in the ileal digestibilities of cheese and beef protein which were 86–89 % (Silvester *et al.* 1997). This suggests that differences between proteins in the present study were due to other causes.

Another finding which has been replicated is attenuation of the DNA-damaging effect of casein by RS (Toden *et al.* 2005). Dietary fibre is important for large bowel function and studies in rodents with experimentally induced cancer have suggested that cereal fibres may be protective (Reddy, 2000). However, human studies have given quite contradictory findings. A large, multicentre prospective European study found a substantial (30 %) reduction in relative risk from the lowest to the highest strata of intake (Bingham *et al.* 2003). In contrast a meta-analysis of a number of prospective studies failed to find any protective effect of fibre (Asano & McLeod, 2002). The expectations of a beneficial effect of fibre came from observations of low risk of colo-rectal cancer in populations who were consuming unrefined foods which were assumed to be high in dietary fibre. Subsequent analysis has shown some of these diets to be actually relatively low in fibre but high in RS (Topping & Clifton, 2001) leading to higher large bowel SCFA. It appears highly possible that protection may be conferred by RS rather than NSP. The present data support this proposal as does the recent observation of a protective effect of RS against experimental cancer in rats (Bauer-Marinovic *et al.* 2006).

Despite the reduction with inclusion of RS, higher levels of DNA damage were found in rats fed soya. The present data are consistent with the increased colonic epithelial cell damage and increased proliferation with rats fed soya protein compared to rats fed casein (Govers *et al.* 1993) and also the increased tumour incidence and burden of dimethylhydrazine-induced intestinal tumour in rats compared to those fed whey or casein (McIntosh *et al.* 1995). The present experimental data are contrary to meta-analyses that suggest that there is an inverse correlation between consumption of non-fermented soya with colo-rectal cancer (Wu *et al.* 2000;

Spector *et al.* 2003). The reasons for this difference are unclear as are those for the generally greater levels of damage with soya seen in this experiment. Minor soya components (e.g. saponins) are known to increase bile acid and steroid excretion (Lee *et al.* 2005) and secondary bile acids are thought to be cytotoxic within the large bowel (Narisawa *et al.* 1974).

The mechanism for the reduction of strand breaks by RS may lie in greater large bowel SCFA availability, especially of butyrate. While SCFA in general are thought to promote a favourable colonic environment, butyrate is thought to be especially important for mucosal integrity both as a metabolic fuel for colonocytes and also through specific actions which promote a normal cell phenotype (Rieger *et al.* 1999; Emenaker & Basson, 2001). The sampling regime of the present experiment precluded the direct measurement of colonic SCFA. However, we did determine caecal and faecal SCFA as indirect measures of production and colonic exposure, respectively. As anticipated, the feeding of RS raised caecal SCFA, consistent with greater fermentation. No consistent relationship was found between the pools of total and individual SCFA and genetic damage. For example, SCFA and butyrate pools were low in rats fed casein and whey in the absence of RS. However, butyrate pools were significantly higher in those fed soya at both levels, i.e. where damage was greater. Similarly, both caecal total SCFA and butyrate were higher in rats fed RS independent of comet tail moment. The present data are similar to those noted earlier in man (Schwartz *et al.* 2002; Muir *et al.* 2004) and animals (Le Leu *et al.* 2002; Anison *et al.* 2003; Bird *et al.* 2004) with various types of RS. Using faecal SCFA as an index of colonic exposure there was a similar lack of relationship between genetic damage and total and individual SCFA. However, faecal SCFA may not be an entirely appropriate measure of exposure as SCFA excretion can be affected by transit as well as production (Topping & Clifton, 2001). *Inter alia* the present data support an interaction between undigested protein, RS and large bowel SCFA (Morita *et al.* 1998; Toden *et al.* 2005). The present data also suggest that the interactions between dietary protein, RS and genetic damage are complex. Other fermentation products and compounds present in the diet may have had a role in damage and/or repair (Rowland *et al.* 1985; Smith & Macfarlane, 1996). In addition, previous studies have suggested that carcinogenic N-nitroso compounds, often found in large quantity in red and processed meat, may also have a strong role in damaging large bowel environment (Cross & Sinha, 2004). Finally, there is a further interaction which remains to be explored, i.e. the microflora. The RS fed in the present experiment is known to be a prebiotic (Topping *et al.* 2003) and has also been shown to modify the activity of pathogenic organisms *in vitro* (Kleessen *et al.* 1997) and so could have led to changes in genetic damage and repair through modifying bacterial activity.

As noted previously (Toden *et al.* 2005), the mucus barrier was thinner in rats fed higher dietary casein in the absence of RS. A similar loss has been seen with high red meat diets (Toden *et al.* 2006) but was not observed with soya or whey. Feeding RS prevented this loss with casein and led to a generally thicker barrier. Previous studies suggest that SCFA increase colonic mucus secretion (Cummings & Macfarlane, 1991; Shimotoyodome *et al.* 2000). Mucins, the

main component of mucus, are encoded by MUC genes and several MUC genes are speculated to have links with colorectal cancer. Recently, Willemsen *et al.* (2003) used cell culture supernatant to demonstrate that SCFA differentially regulates prostaglandin production to stimulate mucin secretion via MUC2 expression in intestinal epithelial cells. However, it is possible also that the microflora may have interacted with diet to affect mucus thickness. Large bowel bacteria are known to possess mucinase activity (Dwarkanath *et al.* 1995) and alterations in this capability could have affected barrier thickness. This is also a potential explanation for the differential effects of whey and soya as neither of these led to the loss of mucus seen with casein. The reversal of the thinning of the mucus barrier by dietary protein by RS warrants further investigation. Loss of barrier function is a feature of inflammatory bowel disease and higher dietary protein has been related to loss of remission from ulcerative colitis (Jowett *et al.* 2004). RS has been shown to protect against experimental colitis (Moreau *et al.* 2003). While doubts have been cast on the relevance of this finding, there is an opportunity to determine the role of interactions between RS and other dietary components in this type of large bowel dysfunction.

The high and low ileal digestibility of the standard and high amylose maize starches, respectively, has been confirmed in rats with an effective ileostomy (Morita *et al.* 1999, 2004). The differences in large bowel variables with RS (greater digesta and faecal mass, lower pH, greater caecal tissue weight) reflect this difference and accord with previous findings (Topping *et al.* 1997; Toden *et al.* 2005). Lower digesta pH is consistent with SCFA production leading to direct acidification and also utilisation of nitrogen (as  $\text{NH}_4^+$ ) for bacterial growth. Faecal pH values reflected those in the caecum, being lower in rats fed RS with values closer to 8 in rats fed the highly digestible starch. Lowering of pH is a possible contributor to some of the changes in genetic damage through reducing the bioavailability of potentially toxic biogenic agents. However, while this factor could have contributed to some of the observed effects, it was not a prime determinant as there was no significant relationship between pH and either genetic damage or mucus layer thickness.

As anticipated from previous studies (Topping *et al.* 1997; Toden *et al.* 2005), the mass of large bowel tissue was higher in rats fed RS than in those consuming the highly digestible starch. The greater length of the colon is also consistent with findings from pigs fed RS (Topping *et al.* 1997). These changes have been explained through a greater availability of SCFA as metabolic fuels.

In conclusion, we have demonstrated that casein, whey and soya proteins have differential effects on colonic DNA damage. Importantly, none of the effects were due to any differences in food intake or body weight. A moderately high level (25%) of whey in the diet did not significantly increase the colonic DNA damage in comparison to 15% whey. Moreover, increased dietary casein and soya, particularly in the absence of RS and its fermentation products, appear to increase DNA damage in a rodent model. It appears likely that dietary proteins interact differently with RS in the colon because the SCFA differed among protein types when fed with RS. However, we have shown that including RS in the diet reduces colonic DNA damage regardless of the types of protein consumed. Further investigations are necessary to

ascertain whether the proteins we have examined and RS exert similar effects in man.

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