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Resistant carbohydrates stimulate cell proliferation and crypt fission in wild-type mice and in the *Apc* $^{Min/+}$ mouse model of intestinal cancer, association with enhanced polyp development

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Fermentation of carbohydrates in the colon can stimulate cell proliferation and could thus be a cancer risk. The effects of resistant carbohydrates, i.e. those not digested and absorbed in the small intestine, on cell proliferation, crypt fission and polyp development were investigated in wild-type and adenomatous polyposis coli multiple intestinal neoplasia (*Apc* $^{Min/+}$) mice. Fifteen 4-week-old female wild-type and fifteen *Apc* $^{Min/+}$ mice were used for each group and fed a chow diet, a semi-synthetic diet or the semi-synthetic supplemented with wheat bran or an apple pomace preparation, both high in resistant carbohydrates, for 8 weeks. Tissue from all mice was used to measure cell proliferation and crypt fission and tissue from the *Apc* $^{Min/+}$ mice was scored for polyp number and tumour burden. There were slight reductions in intestinal mass in the mice fed the semi-synthetic diets and this was increased by the inclusion of resistant carbohydrates. The *Apc* $^{Min/+}$ mice had elevated cell proliferation and crypt fission in the distal small intestine and colon and these were increased by the resistant carbohydrates. Bran or apple pomace significantly increased polyp number in the proximal third of the small intestine. Apple pulp more than doubled polyp number throughout the small bowel (99·2 (SEM 11·1) *v.* 40·0 (SEM 8·2), $P<0{\cdot}004$). Bran and apple pomace increased polyp diameter and hence burden in the colon by 243 and 150 %, respectively ($P<0{\cdot}05$). In conclusion, both types of resistant carbohydrates increased polyp number and tumour burden and this was associated with elevated epithelial cell proliferation and crypt fission.

Dietary fibre: Resistant carbohydrates: Fermentation: Gastrointestinal: Cancer: Cell proliferation: Crypt fission

Up to 90 % of the attributable causes of colon cancer may be environmental, mostly linked with diet and lifestyle[1,2]. Although it was widely believed that high-fibre diets were protective against colorectal cancer, the reality appears to be more complex, and has been the subject of recent controversy. Ten years ago the suggestion that the data underpinning the 'fibre hypothesis' with respect to colon cancer was not as strong as had been implied attracted a lot of criticism[3], especially the assertion that in some circumstances, colonic fermentation of carbohydrates could have adverse effects[4]. Since then several large prospective and intervention studies have shown null effects[5,6], with some showing evidence of increased risk[4,7,8]. Other studies, most prominently a prospective investigation of a large European cohort, have found naturally fibre-rich diets to be associated with reduced risk of colon cancer[9]. Despite the inconsistencies in the evidence relating to colon cancer, there is still general advice from health professionals to increase intake of natural fibre-rich foods because of their overall benefits, including the association with reduced incidence of CVD[10].

Dietary fibre as well as resistant carbohydrate preparations escape digestion in the small intestine and depending on the type can have a range of attributes in the colon including fermentation and bulking. The presence of nutrients in the intestinal lumen, with respect to either luminal nutrition or intestinal workload, has profound actions on the development and maintenance of the intestinal epithelium[11,12]. Atrophy of the colon is observed with a resistant carbohydrate-free 'elemental' diet[13], which can be reversed by resistant carbohydrates, but only in animals with an intestinal flora[14]. This effect is not seen in germ-free rodents[15,16], demonstrating that it is the products of fermentation (the SCFA), rather than bulk, that are trophic. Excessive and rapid fermentation in the colon has been linked to increased proliferation of the intestinal epithelium[15,16]. As increased proliferation is generally considered to be a risk factor for carcinogenesis[17], the desirability of consuming large amounts of rapidly fermented resistant carbohydrates needs to be questioned.

The present study compares the effects of a normal diet, a semi-synthetic diet and the semi-synthetic diet supplemented with resistant carbohydrate preparations of differing fermentabilities. The effects were investigated with normal mice and cancer-prone mice (adenomatous polyposis coli multiple intestinal neoplasia (*Apc* $^{Min/+}$) mice). Intestinal cell renewal and

Abbreviation: *Apc* $^{Min/+}$, adenomatous polyposis coli multiple intestinal neoplasia.

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crypt fission were measured in the small intestine and in the colon[18]. Crypt fission is an alternate means of increasing intestinal tissue mass by creating new crypts and could be the main mechanism for the spread of mutant clones of cells in the gut[19,20]. The present study therefore addresses the effect of different fermentable substrates on cell proliferation and polyp formation as a model for investigating their potential influence on the development of gut cancer.

Methods

The effects of the diets on polyp number size and burden were measured in the *Apc* $^{Min/+}$ mouse, which is generally considered to be a good pre-clinical model of gut cancer[21–23]. The *Apc* $^{Min/+}$ mouse is heterozygous at the *Apc* (adenomatous polyposis coli) locus (as occurs in familial adenomatous polyposis in man) and loss of the remaining wild-type allele leads to β-catenin accumulation and relocation to the nucleus, where it forms a complex with Tcf-4 leading to the transcription of tumour-promoting genes.

Apple pomace, the pulpy material remaining after apples have been pressed for juice extraction, was chosen as it is highly fermentable[24] and bran was chosen as it is lignified and less fermentable, and also because it was the same resistant carbohydrates as used in the Alberts intervention study[8]. The NSP content of the two test materials were measured by the Englyst procedure[25].

Apc *Min/+* mice

Apc $^{Min/+}$ heterozygote mice were originally obtained as a gift from Amy R. Moser (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI, USA)[26]. Male mice were back-crossed to female C57BL/6J mice and the resultant embryos were transferred by aseptic hysterectomy to foster mothers in specific pathogen-free isolators. All breeding was subsequently by brother (C57BL/6J – *Apc* $^{Min/+}$)–sister (C57BL/6J) mating. Genotyping was carried out by a PCR-based method, using three primers including an internal control for normal mouse DNA. On average, half the mice born in a litter will be *Apc* $^{Min/+}$ and half will be wild type. All procedures were approved by the Cancer Research UK Animal Ethics Committees and covered by the appropriate licences under the Home Office Animal Procedures Act, 1986.

Study design

Four groups of fifteen female *Apc* $^{Min/+}$ and fifteen female wild-type littermate mice were put on the following powdered diets prepared by Special Diets Services (Witham, Essex, UK): Group 1, chow diet based a standard mouse maintenance diet (RM1); Group 2, semi-synthetic diet; Group 3, semi-synthetic diet +20 % wheat bran; Group 4, semi-synthetic diet +20 % apple pomace. The bran was supplied by Trouw Nutrition (Witham, Essex, UK) and the dehydrated apple pomace (from fresh apples, variety 'Rome Beauty') was from Kanegrade Ltd (Stevenage, UK).

Autopsy and analysis

After 8 weeks, mice were injected with 1 mg vincristine/kg (to arrest cells as they enter metaphase) and killed 2 h later[27,28]. The small intestines and colons were isolated, rinsed and weighed. The small bowel was divided into three equal sections (proximal, middle, distal), dissected longitudinally using a recently described gut-cutting device[29] and spread on to filter paper. The entire colon was also dissected and spread on to filter paper. These gut preparations were then fixed in Carnoy's fixative for 3 h and then transferred to 70 % ethanol. The tissues were assessed later under a stereomicroscope (×20 magnification) for polyp number and diameter, which was measured using digital callipers. Polyp volume was derived from polyp diameter, assuming a hemispherical shape in the small bowel and a spherical shape in the colon. Tumour burden was calculated as the product of polyp number and polyp volume[30].

Assessment of proliferation and fission throughout the gut was performed using the 'crypt microdissection' method. This method is up to six times faster than scoring histological sections and avoids several problems associated with quantifying histological sections[28].

Representative samples of tissue from the proximal, middle and distal small intestine and colon (taken from positions 10, 50 and 90 % of the total length of the small bowel or colon) were hydrated, hydrolysed and stained with the Feulgen reaction. The mucosal crypts were gently teased apart under a dissection microscope and the numbers of metaphases per crypt (mean of twenty crypts) and crypt fission events per 200 crypts were then determined. All samples were counted in a 'blinded' fashion.

Statistics

Results are presented as the means and their standard errors. Weight and proliferative data were tested by two-way ANOVA: the wild-type and *Apc* $^{Min/+}$ mice were compared so that effects of diet (*v.* the semi-synthetic) and effects of *Apc* $^{Min/+}$ could be revealed and also if there was an interaction between these two factors. Polyp data were tested by one-way ANOVA, and if an effect of treatment was seen, Dunnett's *post hoc* analysis was performed using the semi-synthetic diet as the reference comparison. Minitab Statistical Software (release 10.5 Xtra Minitab, Coventry, UK) was used.

Results

The NSP sugar content of the two preparations is shown in Table 1. The apple pomace contained 23·9 g NSP/100 g

Table 1. NSP constituent sugars (g/100 g)

	Apple pomace	Wheat bran
% DM	92·1	92·2
Rhamnose	0·0	0·0
Fucose	0·0	0·0
Arabinose	4·2	9·2
Xylose	1·3	16·2
Mannose	1·1	0·9
Galactose	1·8	1·3
Glucose	6·8	11·1
Glucuronic acid	0·0	0·9
Galacturonic acid	8·8	0·8
Total	23·9	40·3

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mainly as galacturonic acids, arabinose and galactose from easily fermentable pectin while the bran contained 40·3 % NSP mainly in the form of lignified less fermentable arabinoxylan and cellulose.

There were no differences in body weight between the different groups. The spleens, which are a useful surrogate marker of tumour load, were significantly heavier in the *Apc* $^{Min/+}$ mice when compared to their wild-type littermates, except in the case of the bran-fed group where this was not seen, as reflected by significance effect of *Apc* $^{Min/+}$ status, diet and interaction (Fig. 1).

The small intestines were 10 % shorter and were 20 % lighter in the semi-synthetic groups compared to the chow ($P<0·001$) and showed a modest weight increase of 7 % in the apple pomace-fed group ($P<0·02$). There was also a small effect of *Apc* $^{Min/+}$ status with the small intestines all heavier in the *Apc* $^{Min/+}$ mice (11, 10, 4 and 4 % for the chow semi-synthetic and fibre diets, respectively, $P<0·04–0·001$).

The colons were 15 % shorter and 37 % lighter in the semi-synthetic-fed mice when compared to the chow-fed mice, and were also slightly heavier in the *Apc* $^{Min/+}$ mice. The bran and apple pomace-supplemented diets increased the length and the weights of the colon by 7 and 21 %, respectively ($P<0·015$ and $P<0·001$).

The effects of the various treatments on cell proliferation and crypt fission are shown in Fig. 2. Little difference in proliferation between groups was seen in the proximal small bowel, however, in the distal small intestine and the colon there were marked effects of both diet and *Apc* $^{Min/+}$ status, with the *Apc* $^{Min/+}$ mice having significantly greater metaphase counts compared to the wild-type mice ($P<0·02–0·001$). Proliferation was reduced in the semi-synthetic-fed mice compared to the chow-fed mice ($P<0·02–0·001$). Bran increased proliferation in the distal small intestine and in the colon ($P<0·01–0·001$) while apple pomace only increased proliferation in the colon ($P<0·001$).

Crypt fission in the proximal small intestine was increased in the *Apc* $^{Min/+}$ mice of the chow and semi-synthetic and bran-fed ($P<0·02–0·03$) and was slightly reduced in the bran-fed mice ($P<0·05$), and appeared to be increased in the apple pomace group, which did not demonstrate an effect of *Apc* $^{Min/+}$ status but did have a significant interaction effect ($P<0·05$). A similar pattern was seen in the distal small intestine with significant effects of *Apc* $^{Min/+}$ status being seen in the chow and bran-fed groups ($P<0·03–0·04$) and significant effects of diet were seen in all the groups ($P<0·02–0·001$). No effect of chow on crypt fission was seen in the colon, whereas both resistant carbohydrate-supplemented diets increased fission, especially in the *Apc* $^{Min/+}$ groups. The effect of bran on the *Apc* $^{Min/+}$ mice was particularly marked where there was a 6-fold increase ($P<0·001$) and the apple pomace more than doubled fission (by 120 %, $P<0·03$).

There were no polyps in the wild-type mice so Fig. 3 only shows the result from the *Apc* $^{Min/+}$ mice. There was a significant increase in polyp number in the proximal third of the small intestine with bran, but no effect of bran was seen in the other sites. Polyp counts are lower in the proximal small intestine so that when the results for all the small intestine were pooled no effect of bran was observed. The apple pomace diet was associated with significantly increased polyp number throughout the small intestine (122, 236 and 92 % in the three sites, $P<0·001$) and the total increase was 132 % ($P<0·001$). No effects of the treatments on polyp number were seen in the colon.

Fig. 4 shows the effects of the various diets on polyp diameter and there were no significant effects on diameter in the small intestine, although the diameter of the resistant carbohydrate-fed mice appeared to be smaller (Fig. 5). This thus reduced the significance of the increased polyp number so that the effect of apple pomace on the small bowel burden was reduced to an overall increase of 111 % ($P<0·05$).

Bran and apple pomace both significantly increased polyp diameter in the colon (by 60 and 40 %, $P<0·05$) and when the product of number and diameter (burden) was calculated it can be seen that both types of resistant carbohydrate significantly increased polyp burden in the colon by 243 and 150 %, respectively ($P<0·05$).

Discussion

The results of the present study have allowed us to demonstrate several effects of diet, *Apc* $^{Min/+}$ status and their interrelationships. The *Apc* $^{Min/+}$ mice showed significantly increased cell proliferation and crypt fission when compared to their wild-type littermates. Although the effects of the various diets were more pronounced in the *Apc* $^{Min/+}$ mice, most were still seen in the wild-type which would suggest that the effects reported are not restricted to carriers of germ-line mutations in *Apc*.

There were no differences in polyp count or diameter between the chow and the semi-synthetic-fed mice. This is surprising as the intestines of the semi-synthetic fed mice were significantly shorter and lighter and had lower distal proliferative count. The semi-synthetic-fed mice also had lower fission in the distal small intestine. The present results are compatible with a reduced 'luminal nutrition' or 'intestinal workload' in the distal intestine and colon of the semi-synthetic-fed mice resulting in reduced proliferation rates, which has also been observed in rodents fed resistant carbohydrate-free elemental diets[13].

The resolution of colonic events in the *Apc* $^{Min/+}$ mouse is often rather limited, as there are usually very few polyps in the colon, nonetheless, both types of resistant carbohydrate were associated with significant increases in polyp diameter and this was particularly prominent in the bran-fed mice, which also had markedly increased fission indices in the colon. These mice also had increased proliferation, but so did the chow-fed group and there was no difference in the polyp diameters between the chow and the semi-synthetic group. The conclusion to be drawn is that increased fission in the colon is associated with increased polyp diameter, which then leads to increased polyp burden.

Bran and apple pomace supplementation both increased the number of polyps in the proximal small intestine, and while there are fewer polyps in this part of the small intestine, it may be more responsive to altered diet and growth factor signalling[30,31]. Only the apple pomace was associated with an increased polyp number for the whole small intestine. For bran, no effect was seen in the middle or distal small bowel and it is of interest that the weight of the spleen was not increased in these mice when compared to their wild-type group. Spleen weight can be a useful indicator of tumour

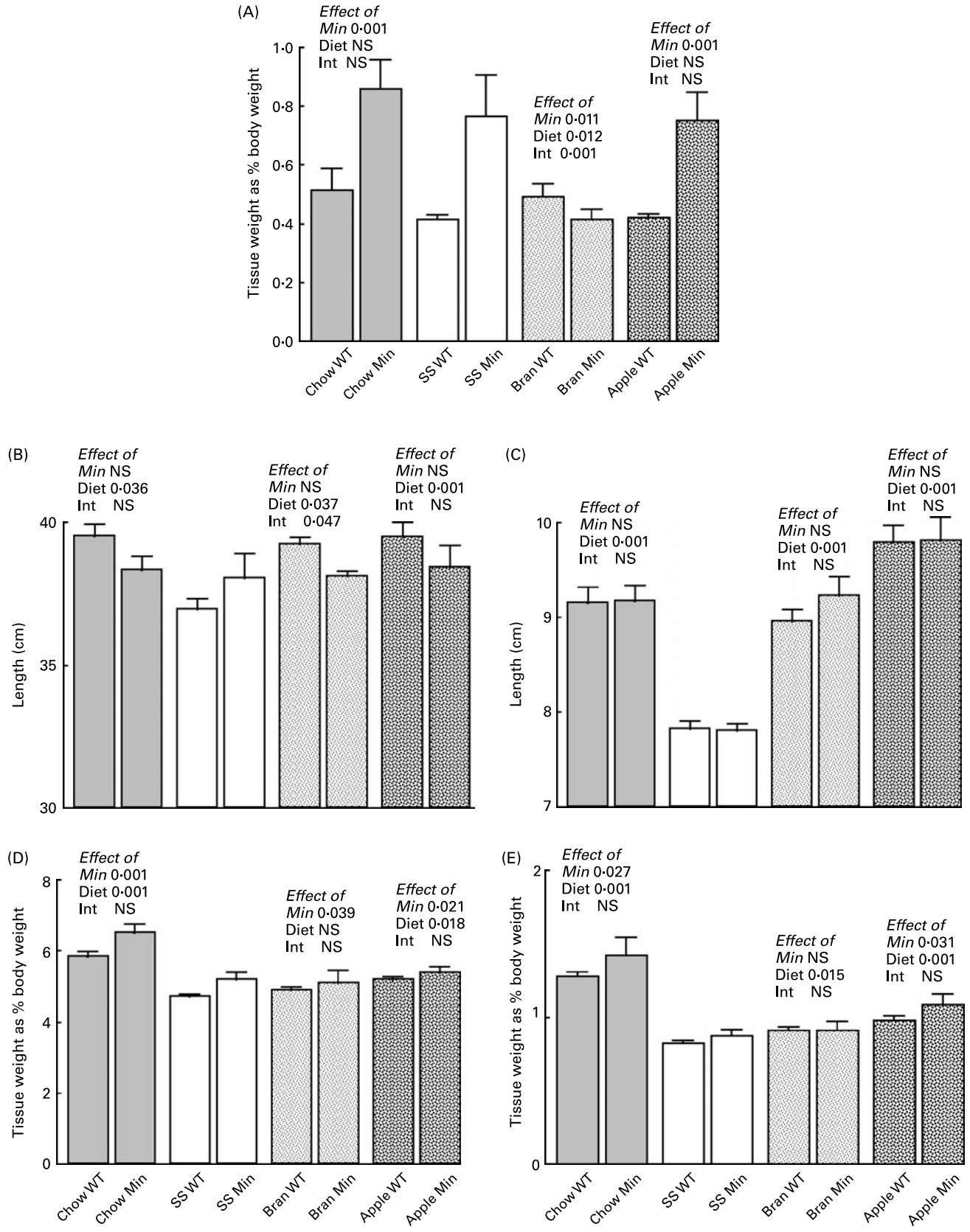

Fig. 1. The effects of the various treatments on tissue wet weight (expressed as a percentage of body weight) for spleen (A), small intestine (D) and colon (E), and on the small intestinal (B) and colonic length (C). Min, multiple intestinal neoplasia (*Apc*$^{Min/+}$) mice; WT, wild-type mice. The results of two-way ANOVA between the semi-synthetic (SS; control) diet and the various dietary modifications are indicated. These analyses test for effects of diet, of *Apc*$^{Min/+}$ status and for interaction effects (int) between Min status and diet.

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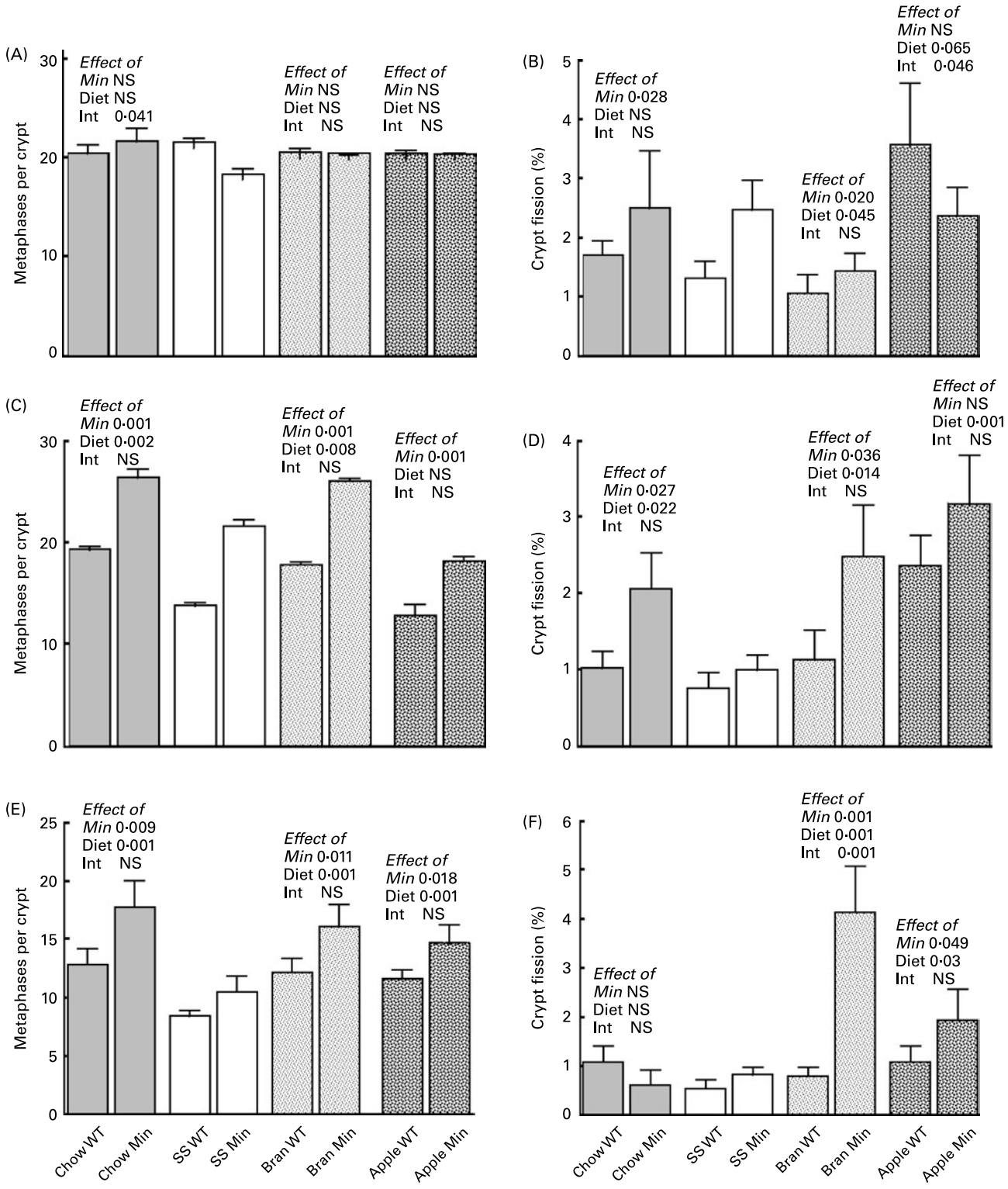

Fig. 2. Cell proliferations, assessed by the 2 h accumulation of vincristine-arrested metaphases per crypt (A, C, E) and crypt fission indices (B, D, F) in the proximal and distal small intestine and mid colon. The sites were defined by their percentage length of the small intestine or colon: ((a, b), 10 % small intestine; (c, d), 90 % small intestine; (e, f), 50 % colon). Min, multiple intestinal neoplasia ($Apc^{Min/+}$) mice; WT, wild-type mice. The results of two-way ANOVA between the semi-synthetic (SS; control) diet and the various dietary modifications are indicated. These analyses test for effects of diet, of $Apc^{Min/+}$ status and for interaction effects (int) between Min status and diet.

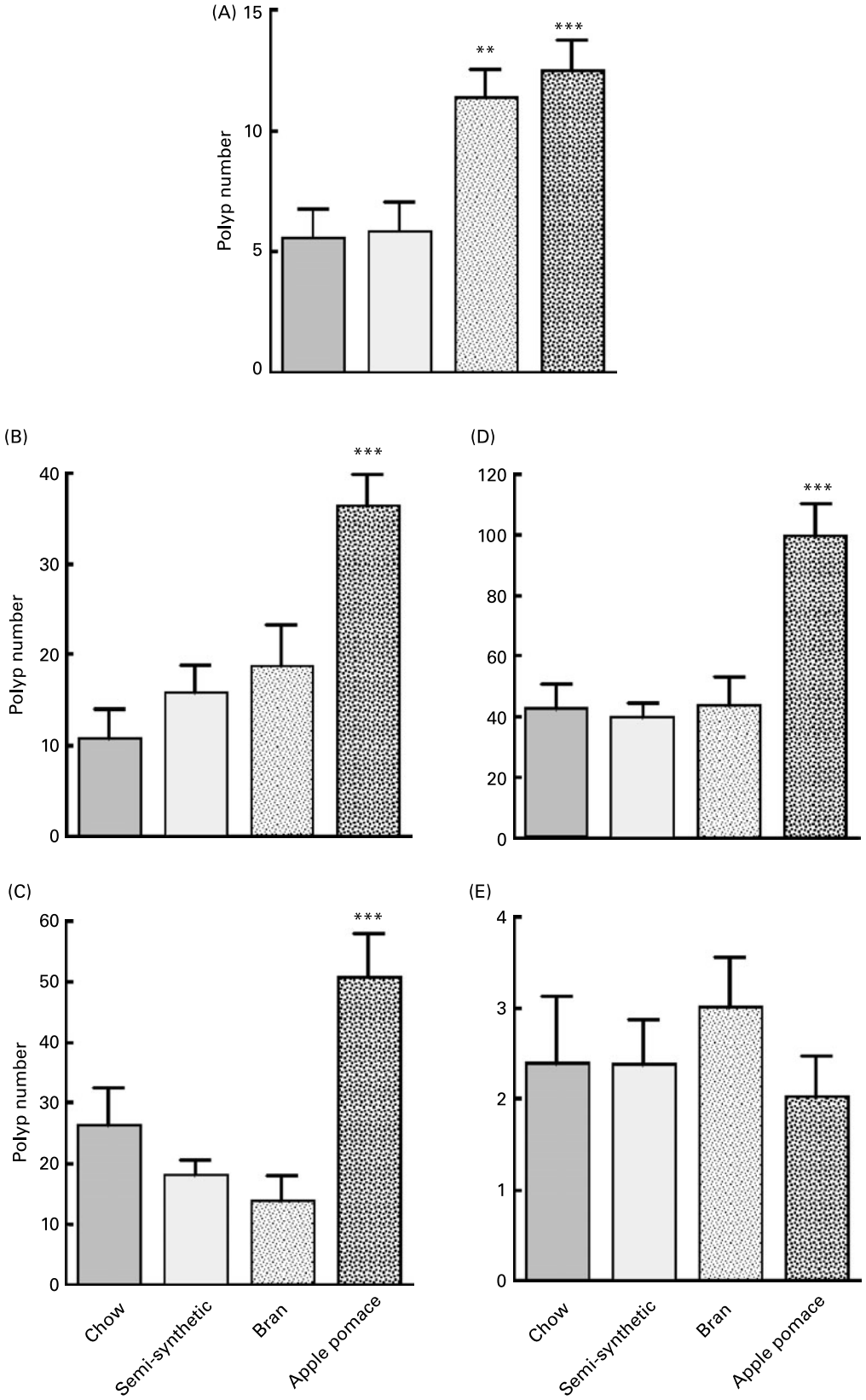

Fig. 3. Polyp number in the three segments of the small intestine (A, proximal; B, middle; C, distal), the three segments together (D) and the colon (E). Values are means with their standard errors depicted by vertical bars. Mean values were significantly different from those of the control (semi-synthetic diet) group (*post hoc* comparison using Dunnett's test after one-way ANOVA): $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$.

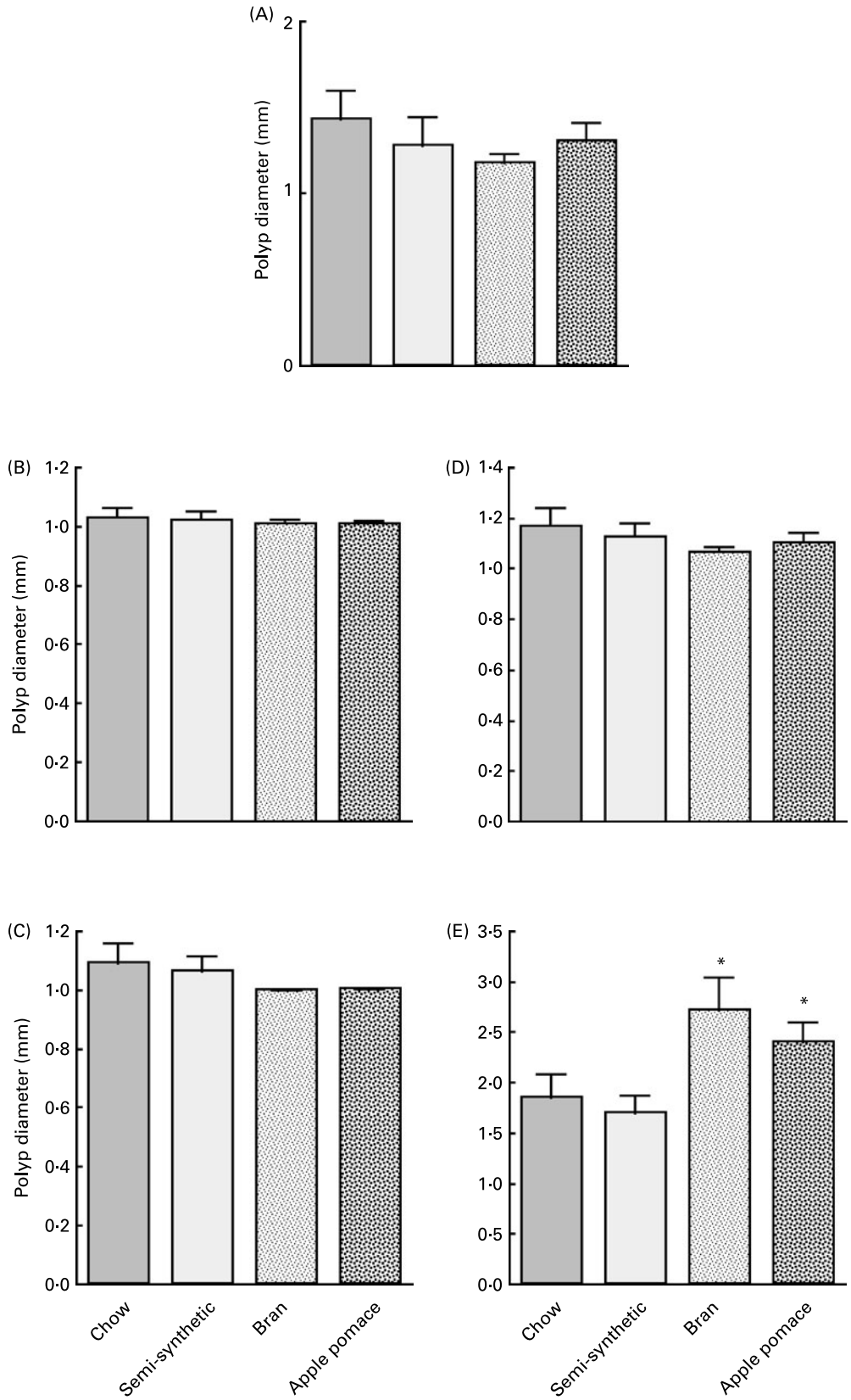

Fig. 4. Polyp diameter in the three segments of the small intestine (A, proximal; B, middle; C, distal), the three segments together (D) and the colon (E). Values are means with their standard errors depicted by vertical bars. Mean values were significantly different from those of the control (semi-synthetic diet) group (*post hoc* comparison using Dunnett's test after one-way ANOVA): *$P<0{\cdot}05$.

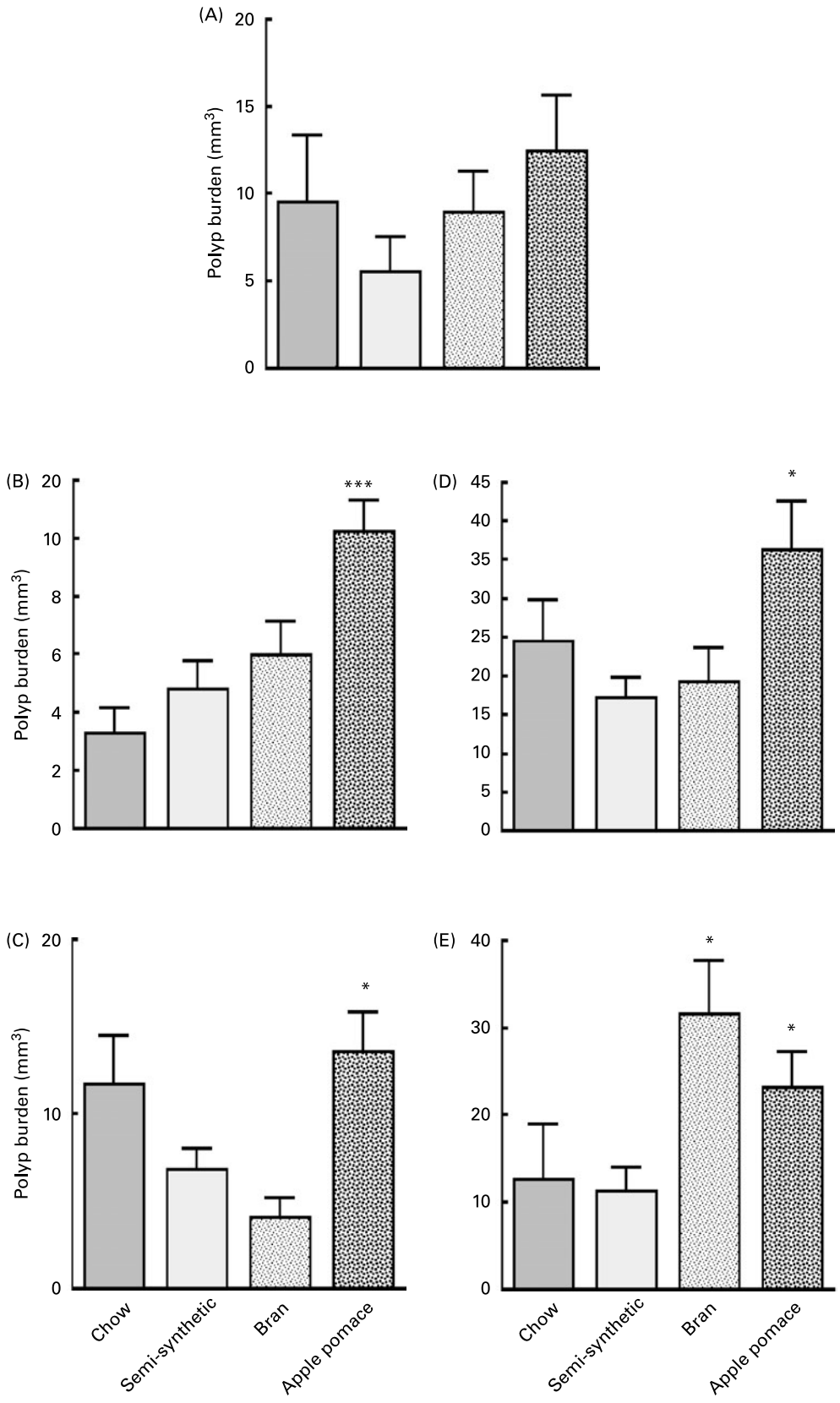

Fig. 5. Tumour burden in the three segments of the small intestine (A, proximal; B, middle; C, distal), the three segments together (D) and the colon (E). Values are means with their standard errors depicted by vertical bars. Mean values were significantly different from those of the control (semi-synthetic diet) group (*post hoc* comparison using Dunnett's test after one-way ANOVA): *$P<0{\cdot}05$, ***$P<0{\cdot}001$.

burden in the *Apc* $^{Min/+}$ mouse[30,32] and may be an indication of intestinal blood loss. The lower spleen weight in this group would thus suggest a protective role of the bran in the distal small intestine and although not significant there did appear to be a reduced polyp count, diameter and burden. The proliferative fission responses of this part of the gut were greater in the bran-fed group and fission was also greater which would indicate that it is only in the colon that increased fission leads to a larger polyp diameter. The role of crypt fission is still unfolding, but it has been proposed to be the main mechanism for the spread of mutant clones of cells in the gut[19,20] and the present results in the colon are compatible with this.

There are many reports on the action of dietary fibre and other resistant carbohydrates on intestinal physiology with most studies showing that increased fermentation leads to increased cell proliferation, which is compatible with the concepts of luminal nutrition or intestinal workload, where the release of SCFA stimulates cell growth. These effects have been questioned, but mainly by those using *in vitro* models, which has led to the concept of the so-called 'butyrate paradox'[33,34]. While *in vitro* models are very useful for studying molecular mechanisms, they may not be appropriate for the study of dietary agents. The gut is a complex multilayered defence system and has many mechanisms to protect its cells from extracellular chemicals, whereas *in vitro* the enterocytes are immersed in them. The results of the present study indicate that crypt fission is an important mechanism for increasing polyp size and they stress the crucial importance of *in vivo* studies.

Other groups have shown increased polyp number in *Apc* $^{Min/+}$ mice with rapidly fermentable resistant carbohydrates and some now even use pectin-fed *Apc* $^{Min/+}$ mice as a model of increased tumour load[35,36]; the same group also found fewer polyps in the distal small intestine of *Apc* $^{Min/+}$ mice fed rye bran[37]. Some of the earlier experiments in laboratory animals, using chemical induction of colon cancer, generally showed a protective effect with supplements of poorly fermentable resistant carbohydrates such as wheat bran or cellulose, while more rapidly fermentable resistant carbohydrate supplements including pectin, oat bran, undegraded carageenan, agar, psyllium, guar gum and alfalfa enhanced tumour development[38]. These earlier findings are a matter of some concern as some rapidly fermentable resistant carbohydrates are now being promoted as 'prebiotics' due to their ability to alter the colonic flora in what is presumed to be a beneficial manner[39,40].

These earlier results suggest that the less fermentable brans may be better, nevertheless it should be remembered that more polyps recurred in women who had had one or more colorectal adenomas removed when given (less fermentable) wheat bran supplements for 3 years[8]. A similar increase in polyp recurrence was also seen in both sexes of similar patients given the more fermentable resistant carbohydrate preparation, ispaghula[41].

The present study has focused on the effects of resistant carbohydrates on proliferation and crypt fission, but there are many other possible mechanisms by which resistant carbohydrates are likely to influence colon tumourigenesis, including several mechanical effects, such as bulking. Depending on the type, different resistant carbohydrates may also soften the stool, reduce intestinal transit, damp glycaemic response, bind carcinogens, bile acids, cholesterol and other potential toxins (but also essential nutrients), and can induce xenobiotic metabolising enzymes. Fermentation of resistant carbohydrates profoundly alters the colonic milieu and the release of SCFA will lower the pH, alter the flora and increase bacterial mass (and hence stool output). This acidification of the colon increases absorption of ferrous iron (the main form in supplements) and there is evidence that ferrous iron was positively associated with distal colon cancer among women who consumed more resistant carbohydrate[42]. Viscous resistant carbohydrates may also have independent actions on mucosal proliferation[43]. There is also the question of whether SCFA and, in particular, butyrate, are 'the preferred fuel' for the colonocyte[44] or whether it is the colonocyte's role to quickly remove these 'toxic' chemicals[4].

The inclusion of rapidly fermentable resistant carbohydrate substrates may perturb the colon; it has been proposed that rapid fermentation could lead to a 'feast or famine' scenario where in the famine the microorganisms must induce enzymes to ferment dying or dead microbes and the colonic epithelial mucosa and mucins. This proteolytic fermentation will generate ammonia and carcinogens, which could increase the probability of precancerous lesions and polyps developing[45].

While both resistant carbohydrate preparations investigated increased cell proliferation, crypt fission and polyps, the effects varied depending on the location in the gut. Although the apple pomace represents a more fermentable substrate, a larger total amount of resistant carbohydrate was provided by the less fermentable bran in the present study, so that the overall fermentation occurring with the two diets was perhaps similar. It seems likely that both the amount and fermentability of resistant carbohydrates have an impact on the gut epithelium function.

The dietary fibre story is still unfolding, and different systems may respond differently, as a recent cohort study has indicated that dietary fibre can prevent breast cancer, but only in pre-menopausal women[46]. Such large-scale intervention studies may eventually lead to a resolution of the role in colorectal cancer, but it seems that they are very susceptible to the actions of covariates. For example, a large meta-analysis initially showed that dietary fibre could be protective, but further analyses accounting for other dietary risk factors removed the association[6]. These methods were then used on the European Prospective Investigation into Cancer and Nutrition (EPIC) data and the 40 % reduction in risk associated with fibre[9] was removed[47], although this has been recently challenged[48].

Nevertheless, the general advice from health professionals to consume a natural high-fibre diet of fruit, vegetables and whole-grain products[49] is fully supportable, as there is convincing evidence that such diets are beneficial with respect to obesity, CVD, diabetes and some types of cancer[10,50,51]. In addition, natural fibre-rich diets, for which dietary fibre defined as 'intrinsic plant cell wall polysaccharides' is a good marker[52], are likely to contain co-passengers, such as the many different phytochemicals, which have been shown to exert effects on cell proliferation throughout the alimentary tract[53].

The present study supports the hypothesis that fermentation of large amounts of resistant carbohydrates by gut bacteria

may have potentially detrimental effects on colonic health. There is little evidence to suggest that the resistant carbohydrates in the amounts present in a natural fibre-rich diet represents a cancer risk. However, there is potential for concern if easily fermentable resistant carbohydrate preparations are consumed in large amounts and therefore these types of functional ingredients, including resistant oligosaccharides and resistant starch, should be researched for both short- and longer-term effects and, if shown beneficial to health, promoted individually.

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