

Only fibres promoting a stable butyrate producing colonic ecosystem decrease the rate of aberrant crypt foci in rats

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Abstract

Background—Dietary fibres have been proposed as protective agents against colon cancer but results of both epidemiological and experimental studies are inconclusive.

Aims—Hypothesising that protection against colon cancer may be restricted to butyrate producing fibres, we investigated the factors needed for long term stable butyrate production and its relation to susceptibility to colon cancer.

Methods—A two part randomised blinded study in rats, mimicking a prospective study in humans, was performed using a low fibre control diet (CD) and three high fibre diets: starch free wheat bran (WB), type III resistant starch (RS), and short chain fructo-oligosaccharides (FOS). Using a randomised block design, 96 inbred rats were fed for two, 16, 30, or 44 days to determine the period of adaptation to the diets, fermentation profiles, and effects on the colon, including mucosal proliferation on day 44. Subsequently, 36 rats fed the same diets for 44 days were injected with azoxymethane and checked for aberrant crypt foci 30 days later.

Results—After fermentation had stabilised (44 days), only RS and FOS produced large amounts of butyrate, with a trophic effect in the large intestine. No difference in mucosal proliferation between the diets was noted at this time. In the subsequent experiment one month later, fewer aberrant crypt foci were present in rats fed high butyrate producing diets (RS, $p=0.022$; FOS, $p=0.043$).

Conclusion—A stable butyrate producing colonic ecosystem related to selected fibres appears to be less conducive to colon carcinogenesis.

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Keywords: fibre; fermentation; butyrate; colon carcinogenesis; aberrant crypt foci; rat

Although it had been suggested that dietary fibres can protect against colon cancer,^{1–4} the results are debatable and prevention programmes have been limited to general lifestyle guidelines.⁵ Classically defined as non-starch polysaccharides, fibres now include other sources of fermentable substrate for microflora, such as resistant starches, oligosaccharides, and endogenous substrates.⁶ According to the origin of their partial resistance to α

amylase, resistant starches have been classified into type I (physically inaccessible), type II (semicrystalline structure), and type III (retrograded starch).⁷ Among fibres, carbohydrates producing large amounts of butyrate appear to be of greatest interest as butyrate is an energy yielding substrate for colonocytes, affects cellular function, is an antineoplastic agent in vitro, and has been implicated in the protective effect of fibre in rodents.^{3–8–9} However, some in vivo studies have shown no protection, even with known butyrate producing fibres. These conflicting results may relate to the heterogeneity of the fibre and basal diet, feeding protocol, animal model, chosen biomarker, and/or stage of colon carcinogenesis.

To clarify this issue, we focused on the butyrate hypothesis and the initiation stage. To control factors other than butyrate, the study was planned both for fibre source and in vivo parameters. Sources were wheat bran, resistant starch, and fructo-oligosaccharides. Wheat bran produces high concentrations of butyrate^{10–11} and was protective in animal studies.^{3–4} However, it is a mixture of proteins, lignin, cellulose, hemicelluloses, and entrapped starch.¹² As starch produces butyrate,^{13–14} wheat bran was rendered starch free. The effect of starch itself was monitored by type III resistant starch, and the effects of butyrate were distinguished from those related to the physico-chemical characteristics of starch by use of another butyrate producing fibre, short chain fructo-oligosaccharides.^{6–15} An 8% level of fibre was chosen, sufficient to produce a physiological effect without inhibiting growth¹⁶ and approximating the highest level found in Western diets.³ Moreover, 8% wheat bran was protective against carcinogenesis in rats.⁴ The control diet was low fibre to allow normal intestinal transit and avoid mucosal atrophy.¹⁷ The basal diet was not high fat to avoid a promoting effect not within the scope of the study. We did not use sucrose but digestible starch to balance the diets, as sucrose increases colonocyte proliferation and susceptibility to initiation.¹⁸ Although studies characterising their fermentation have shown that the colonic ecosystem needs time to adapt to fibres,^{19–21} most experiments on carcinogenesis have involved a

Abbreviations used in this paper: AC, aberrant crypt; ACF, aberrant crypt foci; AOM, azoxymethane; CD, low fibre control diet; FOS, short chain fructo-oligosaccharide enriched diet; PCNA, proliferating cell nuclear antigen; RS, type III resistant starch enriched diet; SCFA, short chain fatty acid; WB, starch free wheat bran enriched diet.

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Table 1 Composition (g) of the experimental powdered diets (low fibre control diet (CD), starch free wheat bran enriched diet (WB), type III resistant starch enriched diet (RS), and short chain fructo-oligosaccharide enriched diet (FOS))

	CD	WB	RS	FOS
Starch free wheat bran ^a	0	7.60	0	0
Retrograded high amylose corn starch ^a	0	0	19.95	0
Short chain fructo-oligosaccharides ^a	0	0	0	6.00
Pregelatinised starch ^b	64.27	63.20	50.32	61.12
Cellulose	2.00	2.00	2.00	2.00
Casein	20.00	20.00	20.00	20.00
DL-methionine	0.40	0.40	0.40	0.40
Corn oil	2.00	2.00	2.00	2.00
Lard	6.33	6.33	6.33	6.33
Minerals ^c	4.50	4.50	4.50	4.50
Vitamins ^c	0.50	0.50	0.50	0.50

The control diet was a low fibre diet containing 2% cellulose.

^aEach fibre source provided 5.8% (dry matter) indigestible carbohydrates in addition to cellulose. Starch free wheat bran contained 78.93% total fibre (digestibility 34.33%) and 0.51% remaining starch. Type III resistant starch provided 30.07% indigestible starch. FOS consisted only of indigestible carbohydrates, the difference between the amount of FOS and indigestible carbohydrates provided corresponding to the moisture of the fibre.

^bThe amount of pregelatinised digestible starch was calculated so that the sum of the fibre source plus digestible starch provided 257.1 kcal/g dry matter, as did the 64.27 g pregelatinised starch of the control diet. The energy contribution of the fibre sources was determined as 0.56 kcal/g dry matter for starch free wheat bran, 4 kcal/g dry matter for digestible starch, and 2.1 kcal/g dry matter for FOS.

^cINRA formula No 102.

very short (if any) period of adaptation before injection of the carcinogen. Thus we determined the time needed to achieve stabilisation before evaluating the protective effect of fibres with aberrant crypt foci (ACF), one of the most reliable intermediate biomarkers of colon cancer.^{4 22-30} ACF were induced by azoxymethane (AOM), which cannot be adsorbed by fibres,³¹ which would have interfered with the study of the effects of butyrate alone.

Materials and methods

STUDY DESIGN

This two part study was carried out on 10 week old rats. The first set of experiments estimated the time needed for adaptation to fibre and stabilisation of the colonic ecosystem. Samples of large intestine were harvested on day 44 to check the proliferation status at the time chosen for induction of ACF. In the second set of experiments, rats fed the same diets were injected with AOM on day 44. All analyses were performed blind: only the person in charge of animal care knew the diet allocations. Codes were broken only for statistical analysis.

EXPERIMENTAL DIETS (TABLE 1)

Diets used before rats were fed experimental diets were from UAR (Villemoisson-sur-Orge, France). Cellulose was Arbocel type B00 (Durieux, Marne-la-Vallée, France). Wheat bran was rendered starch free using an enzymatic method.³² Type III resistant starch (Cerestar) was a retrograded high amylose corn starch. Short chain fructo-oligosaccharides (glucose-fructose, n=4) were produced from sucrose using a fungal fructosyltransferase (Actilight P, Béghin-Meiji Industries, Neuilly-sur-Seine, France). Indigestible carbohydrate was analysed as total dietary fibre³³ in wheat bran, and as resistant starch³⁴ in corn starch.

ANIMALS

Inbred BDIX rats (Iffa-Credo, L'Arbresle, France) were chosen to minimise interanimal variance. Rats bred in our facilities were used

for the first experiment, allowing randomisation of the animals with blocking factors and their staggered inclusion. Rats were housed in suspended stainless steel wired cages. Diet and tap water were provided ad libitum. All animal handling procedures were done in accordance with the rules of the French Ministry of Agriculture (agreement No A44565).

PROTOCOL FOR THE FIRST EXPERIMENT: STUDY OF FIBRE FERMENTATION (FIG 1)

The 96 rats were randomised in blocks of four rats, controlling the variability related to age, sex, parents, and ante/perinatal epigenetic factors. At 8–10 weeks of age, rats received the experimental diets. All subsequent procedures (except feeding) were performed blind. Animal growth was estimated from relative weights, that is weight gain from week 1, calculated as follows: weight gain at week x = (weight at week x – weight at week 1)/weight at week 1.

SAMPLE HARVESTING FOR THE FIRST EXPERIMENT

The large intestine was isolated by tying the ileocecal and anorectal junctions and then removed and weighed. The length of the full large intestine was measured from the caecal apex to the anorectal junction, using a vertical measure with a constant 10 g tension. The three large intestine segments (caecum, and proximal and distal colon) were tied to isolate the contents. The middle of the colon roughly defined the junction between the proximal and distal colon. The full segments were separated, weighed, and put on ice. Three aliquots of content per segment were isolated in the following order of priority: short chain fatty acid (SCFA), residual starch, and lactate assays. Mucosal samples were harvested for immunohistochemical studies.

ASSAY OF FERMENTATION PRODUCTS

SCFA concentrations were measured by gas chromatography (Delsi 300, Argenteuil, France) using a Chromosorb W-AW 60-80 mesh column (Saint-Quentin Fallavier, France).³⁵ Residual starch was analysed using an enzymatic method.³⁴ Lactic acid was quantified by a UV enzymatic method (Boehringer, Mannheim, Germany).

IMMUNOHISTOCHEMISTRY AND PROLIFERATION INDEXES

Samples were labelled with peroxidase labelled monoclonal mouse antiproliferating cell nuclear antigen (PCNA) antibody (Dako, Carpinteria, California, USA). Measurements were performed with the AxioHome system (Zeiss, Jena, Germany).

PROTOCOL FOR THE SECOND EXPERIMENT: INDUCTION OF ACF WITH AOM

Thirty six rats were fed powdered experimental diets for 44 days and were then injected subcutaneously with AOM (Sigma, St Quentin, France) using the standard procedure.²¹ Diets were continued until sacrifice to avoid any uncontrolled disturbance of the colonic ecosystem. Rats were weighed once a week from D0 until sacrifice one month later.

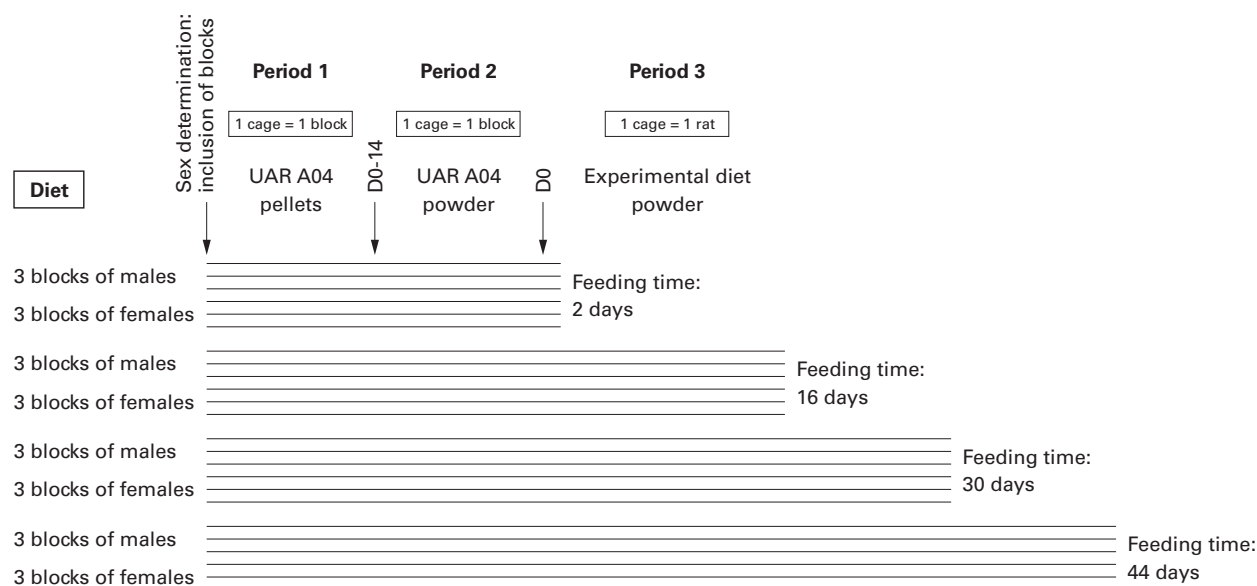


Figure 1 Protocol for the study of experimental diet fermentation. Rats ($n=96$) were randomised before the start of the experiment into 24 blocks (six blocks for each feeding period), one block representing a group of four rats of the same litter and sex. Staggered inclusion of blocks allowed the processing of only one block per sacrifice day, within a period (about one hour) short enough to ensure that all contents could be considered as at the same stage of fermentation. As a possible “experimenter effect” related to the long study period could not be eliminated, inclusion of the 24 blocks was randomised so that all sacrifice days were determined before the experiment began. Blocks were included (median age 46 days) after sex determination and according to these criteria. The four rats of each block, housed in a single cage, were fed successively A03 breeding diet and A04 maintenance diet (UAR). Two weeks before day 0 (D0), rats were housed one per cage and fed powdered maintenance diet. At D0 (median age 72 days), each animal from a block received one of the four experimental diets (table of permuted randomised blocks) and underwent the feeding period allocated by randomisation. Animals were weighed weekly throughout the experiment, from week 1 (at D2) to the day of sacrifice: D16, D30, or D44. Previous studies showed that short chain fatty acid concentrations increased following consumption of the meal and then stabilised during the 8–12 hour postprandial period. Even fed *ad libitum*, rats had the highest consumption of food at the beginning of the dark period. Rats were thus sacrificed 10 hours later, one block at a time, in the order of their codes.

COUNTING OF ABERRANT CRYPT FOCI

ACF were scored blindly twice, by two observers, using the classical procedure, aberrant crypts (AC) being distinguished by their slit-like opening, increased staining, size, pericryptal zone, and slight elevation compared with normal crypts.^{25–36} Homogeneity of records from both observers was controlled using Pearson’s correlation. ACs are rare, and a single AC is not always easily distinguishable from a normal crypt. To favour specificity, AC and ACF values for each segment of rat colon (proximal colon, and upper and lower distal colon) were the minimum count, regardless of the observer, thereby reducing the risk of false positives. The total numbers of AC and ACF per rat were calculated as the sum of these constructed values from each segment.

STATISTICAL ANALYSES

Data were studied in the context of the general linear model, either ranked or transformed values being used when needed.^{37–39} Interactions were included in the models, except when the block factor was used. For analyses of variance (ANOVA), comparisons of means were performed with Fisher’s least significant difference test with Bonferroni’s correction or, for repeated measures analyses, with orthogonal contrasts comparing each of the fibre enriched diets to the low fibre diet. As fermentation was considered as a whole, with tightly correlated data relating to the metabolism of one fibre by a unique microflora throughout the large intestine of a single rat, mixed models of ANOVA with repeated measures were applied to the spatially correlated data⁴⁰ of fermentation from each rat (referred to as the vector of one given

SCFA), that is SCFA concentrations from the caecum, and proximal and distal colon. The models used are given close to the results, or with their illustration. All tests were two tailed, and statistical significance was assigned at $p<0.05$. Analyses were done with Systat 5.2.1 for the Macintosh (Evanston, Illinois, USA).

Results

CHANGES OVER TIME IN THE FERMENTATION OF EACH EXPERIMENTAL DIET (FIGS 2, 3)

Analyses were performed on ranked data as variances differed considerably according to the feeding period and block. As the sex effect was not significant, it was not included in the models. When the time effect was significant, orthogonal contrasts were run to compare D2 with later times (coded $D \geq 16$). When this test was non-significant, changes along the four time periods were explored using linear contrasts (monotonic relationship). Global trends emerged from statistical analyses. First, variances were high at D16 and D30, probably because of the transition period in adapting to diets, and then decreased at D44. Secondly, changes in fermentation of a given diet over time were especially qualitative.

Fermentation of the low fibre control diet (CD) was homogeneous over time. A monotonic decrease in acetate ($p=0.005$) and a monotonic increase in butyrate ($p=0.03$) was observed only in the caecum. Fermentation of the starch free wheat bran enriched diet (WB) led to an increase in butyrate from D2 to D16 in the caecum ($p=0.01$) and proximal colon ($p=0.03$), and then a progressive decrease to a level on D44 close to that on D2. In rats fed the type III resistant starch enriched diet (RS), the

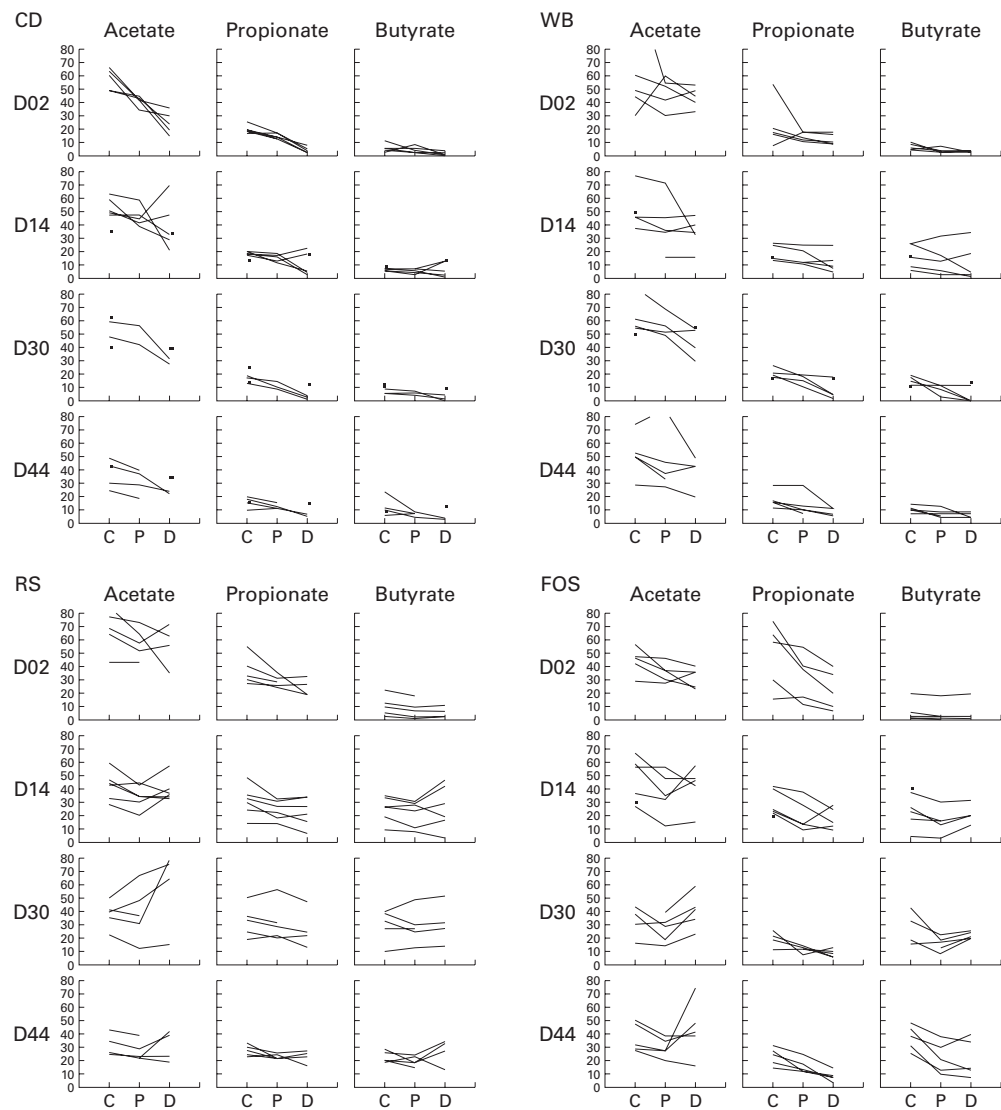


Figure 2 Changes over time in the fermentation of the four diets (low fibre control diet (CD), starch free wheat bran enriched diet (WB), type III resistant starch enriched diet (RS), and short chain fructo-oligosaccharide enriched diet (FOS)) along the large intestine. Twenty one blocks (84 rats) instead of 24 were used for this part of the study as some samples were lost. On the y axis are the concentrations of short chain fatty acids (acetate, propionate, and butyrate), expressed in $\mu\text{mol/g}$ wet content. Values from the caecum (C), and proximal (P) and distal (D) colon of each rat are linked together, each line thus representing the individual fermentation pattern along the large intestine of one rat. When there were missing values for proximal colon concentrations (low content), points were plotted to mark the concentrations in the caecum and distal colon, but not linked.

main modifications occurred between D2 and D \geq 16: compared with D2, acetate decreased in the caecum ($p=0.001$) and proximal colon ($p=0.003$), while butyrate increased in the caecum ($p=0.02$), and proximal ($p=0.007$) and distal colon ($p=0.009$), leading to an equilibrium between the three major SCFAs after D16. Only caecal acetate was modified further, decreasing monotonically from D16 to D44 ($p=0.03$). Total SCFAs decreased monotonically over time in the caecum ($p=0.01$) and proximal colon ($p=0.02$), paralleling the decrease in acetate. No modifications were noted for propionate. These major qualitative changes suggested that an adaptive period was needed which was defined by measuring residual starch in the caecum. Throughout the experiment, butyrate was negatively correlated with residual starch ($p<0.001$) which decreased over time (fig 4). The variance in

residual starch for RS was similar to that of other diets only at D44. Fermentation of the short chain fructo-oligosaccharide enriched diet (FOS) produced a large amount of propionate, and then progressively of butyrate. There were no modifications in total SCFAs but only qualitative ones concerning propionate and butyrate. A monotonic decrease in propionate was observed from D2 to D30 in all segments (p values 0.03–0.06), whereas it was stable from D30 to D44. From D2 to D44, butyrate increased monotonically in all segments (p values from 0.001 to 0.02), the main changes between D16 and D44 concerning the caecum (fig 5). Lactate accumulation differed in the short and long term (table 2). Changes in fermentation over time were thus associated with all fibre enriched diets. WB showed a transient increase in butyrate concentration in the upper large intestine, RS produced high

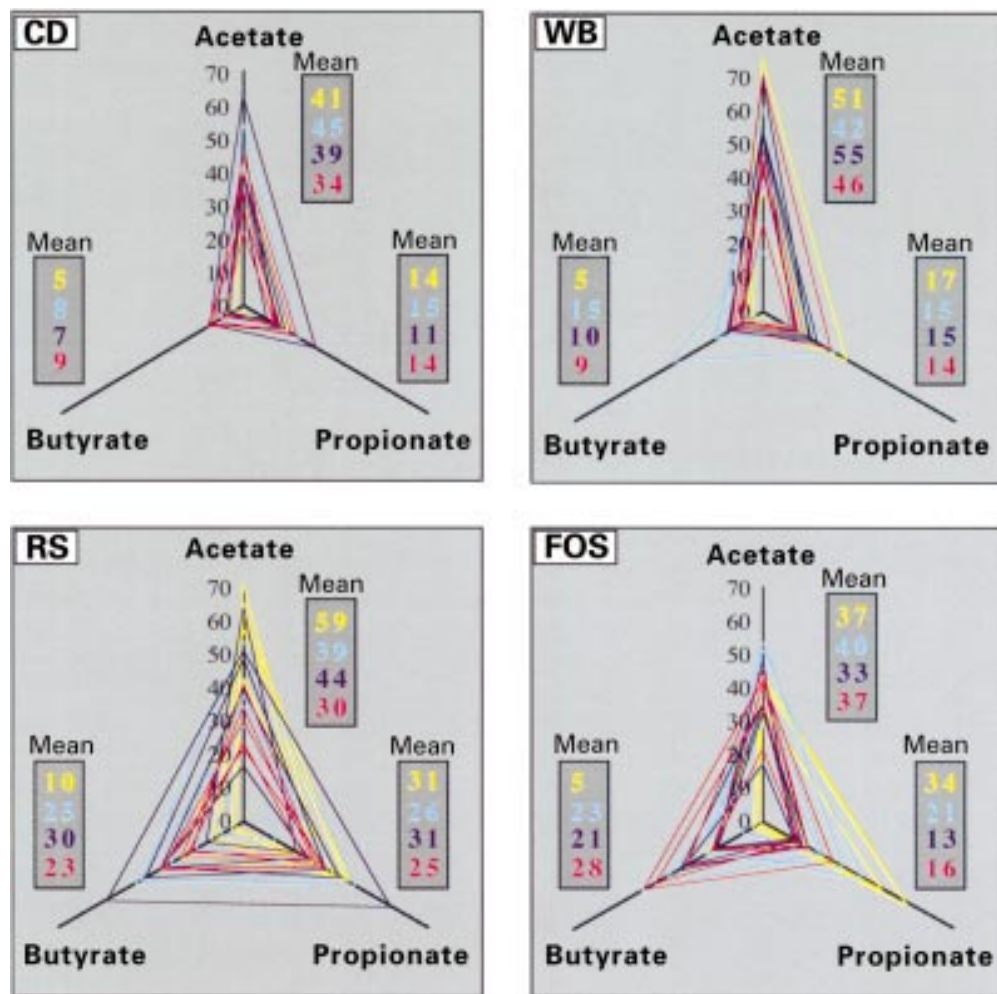


Figure 3 Changes over time in the relationships between the major short chain fatty acids (SCFAs) issued from fermentation of the four diets (low fibre control diet (CD), starch free wheat bran enriched diet (WB), type III resistant starch enriched diet (RS), and short chain fructo-oligosaccharide enriched diet (FOS)). The axes indicate individual mean large intestine concentrations (an average of caecum, and proximal and distal colon concentrations, expressed in $\mu\text{mol/g wet content}$) of acetate, propionate, and butyrate for each rat. These values are linked to form a triangle representing the mean fermentation pattern of the rat. The triangle area is proportional to global SCFA production, and the ratio (relative concentration) of each SCFA can be determined from the shape of the triangle, regardless of its size: the more acute the angle, the higher the ratio. Each dark gray box indicates the mean values of one SCFA concentration for a given time and diet. Colour coding for feeding periods is yellow for D2, blue for D16, dark blue for D30, and red for D44.

concentrations of butyrate throughout the large intestine after D16, and FOS led to high concentrations of propionate and lactate in the short term, and of butyrate in the long term (fig 5).

COMPARISON OF FIBRE ENRICHED DIETS WITH THE LOW FIBRE DIET FOR EACH FEEDING PERIOD
 The model $vector = diet + block$ was used, with a variance stabilising transformation ($Y = \log(SCFA \text{ concentration} + 1)$). Fermentation of WB produced more acetate ($p = 0.007$) and propionate ($p = 0.01$) in the distal colon at D2, and more butyrate in the caecum at D16 (NS) and D30 ($p = 0.05$). On D44 however, WB fermentation was similar to that of CD. Fermentation of RS was qualitatively similar (relative concentrations) to that of CD at D2. Both acetate and propionate were higher in the proximal ($p = 0.01$) and distal ($p = 0.007$) colon. The higher concentration of butyrate was not significant. For longer feeding periods, RS produced more butyrate (p values 0.5–0.005 depending on the segment and feeding period)

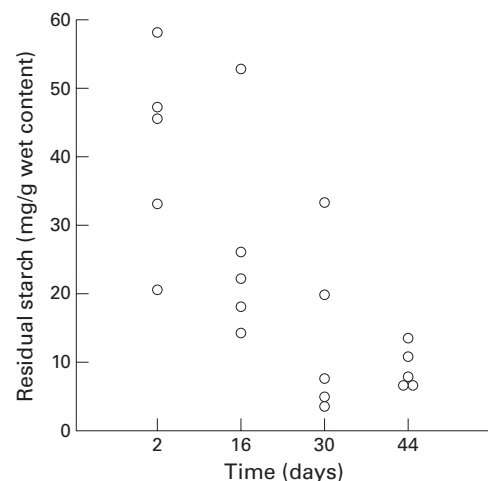


Figure 4 Residual starch concentration in the caecum of rats fed the resistant starch enriched diet (RS) for 2, 16, 30, or 44 days. Each point represents one rat.

and propionate (only on D30 and D44; p values 0.03–0.004). Fermentation of FOS was

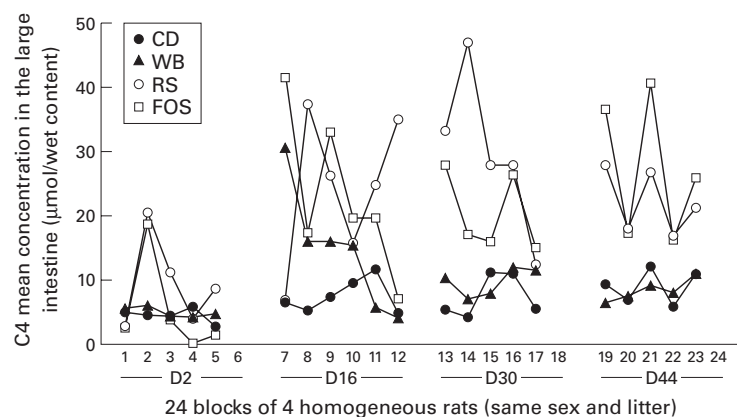


Figure 5 Time and interindividual effects on the mean butyrate large intestine concentration ($\mu\text{mol/g}$ wet content) in rats from the 21 blocks (four rats of the same litter, sex, and feeding period) in this part of the study. The four rats of one block are on the same vertical. To facilitate interpretation, lines have been drawn linking the butyrate concentrations of animals fed the same diet for a given period. On D44, mean butyrate concentrations were higher for the short chain fructo-oligosaccharide enriched diet (FOS) than for the type III resistant starch enriched diet (RS), but this was only due to higher caecal concentrations.

Table 2 Lactate concentration in rats fed experimental diets (low fibre control diet (CD), starch free wheat bran enriched diet (WB), type III resistant starch enriched diet (RS), and short chain fructo-oligosaccharide enriched diet (FOS)) for 2 (D2) or 44 (D44) days

	CD	WB	RS	FOS
D2				
Caecum	16.3 (8.4–24.3) n=5	12.6 (8.7–15.5) n=5	9.3 (4.5–21.6) n=5	76.1 (25.6–77.2) n=5
Colon	5.7 (ND) n=1	9.4 (3.3–15.6) n=4	8.0 (5.1–28.3) n=3	39.1 (33.8–44.8) n=3
D44				
Caecum	11.9 (8.5–28.4) n=5	21.4 (11.3–28.4) n=5	11.1 (4.9–20.9) n=4	22.4 (19.5–34.6) n=5
Colon	7.1 (ND) n=1	8.7 (8.2–10.1) n=3	7.5 (3.2–21.9) n=4	21.2 (9.6–39.2) n=4

Results are expressed as median (min–max) of the sum of D-lactate and L-lactate concentrations ($\mu\text{mol/g}$ wet content) measured in contents of rats for which a sufficient amount remained after aliquoting for short chain fatty acid and residual starch assays. As concentrations of D- and L-lactate isomers were similar, only total concentrations are indicated.

Statistical analyses (ANOVAs on ranked data followed by Bonferroni's test) were only performed for the caecum, using the model: $Y = \text{diet} + \text{block}$. At D2, FOS was significantly different from CD ($p = 0.017$); at D44, the diet effect was not significant.

Table 3 Crypt depth and proliferation status in rats fed the experimental diets (low fibre control diet (CD), starch free wheat bran enriched diet (WB), type III resistant starch enriched diet (RS), and short chain fructo-oligosaccharide enriched diet (FOS)) for 44 days (median (min–max))

	CD	WB	RS	FOS
Crypt depth (μm)				
Caecum	143 (125–150)	144 (135–180)	162 (154–177)	162 (149–174)
Colon	282 (221–297)	324 (231–350)	295 (234–303)	264 (221–277)
Rectum	191 (184–213)	224 (191–252)	221 (211–289)	215 (204–225)
Count of PCNA+ cells relative to crypt depth				
Caecum	0.91 (0.72–1.41)	0.95 (0.68–1.97)	0.82 (0.78–1.05)	0.90 (0.62–1.23)
Colon	1.42 (1.06–2.51)	1.33 (0.78–2.53)	2.10 (1.54–2.83)	1.88 (1.88–1.98)
Rectum	3.56 (3.50–3.61)	2.15 (1.12–5.68)	1.81 (0.35–2.82)	3.12 (3.05–3.62)
Height of the proliferative zone (% crypt depth)				
Caecum	36 (30–52)	36 (25–47)	36 (26–54)	37 (25–46)
Colon	40 (29–47)	33 (31–49)	45 (39–52)	41 (30–44)
Rectum	31 (30–31)	26 (16–39)	25 (21–29)	28 (26–35)

For each rat ($n=16$, four blocks), samples labelled with antiproliferating cell nuclear antigen (PCNA) were issued from the caecum (caecal apex), colon (upper part of the distal colon), and rectum (end of the distal colon). Only crypts with a lumen visible from bottom to top were taken into account, and 30–50 crypts per large intestine segment were analysed. All observations were performed blind. The measurements were the depth of the crypt (upper limit: just under the surface epithelium), the number of PCNA positive cells per crypt, and the height of the proliferative zone (upper limit: top of the highest labelled cell). Two proliferation indexes were constructed: ratio of positive cells to crypt depth, and height of the proliferative zone relative to crypt depth. For each rat, the mean values for observed crypts were determined per segment.

Analyses of the effect of diet were performed independently for the caecum, colon, and rectum. Ranked data were analysed using the ANOVA model: $Y = \text{diet} + \text{block}$, followed by Bonferroni's test. In the caecum, crypts were deeper with RS ($p = 0.048$) and FOS ($p = 0.078$, NS) than with CD. No proliferation index was statistically different between fibre enriched diets and CD. The proliferative zone was always limited to the lowest 60% of crypts (maximum height 54%).

also different from that of CD, with more propionate on D2 (p values 0.03–0.002), and more butyrate from D16. On D16, butyrate was higher only in the caecum ($p=0.03$), and on D30 and D44 in all segments (p values 0.008–0.003).

Although inbred rats were used, production of butyrate within the blocks did not become homogeneous before D44 (fig 4). Furthermore, some yet undetermined factors in individual rats influenced the intensity of the response to diets: on D44, differences between high and low butyrate producing diets were proportional to the “basal” level of butyrate production (CD) within a given block (fig 4). Because of this observation, and because variances were lowest on D44 (time-effect on fermentation, residual starch concentration), D44 was chosen to study the effects of diet on the colon mucosa (proliferation and susceptibility to carcinogen).

EFFECT OF DIETS ON MORPHOMETRICS AND MUCOSA PROLIFERATION

Regardless of sex and duration of diet, weight gain (repeated measures analyses) and weight at sacrifice (model: $\text{weight} = \text{diet} + \text{time}$) showed no dependence on diet. On D2 there were no differences according to diets for either large intestine length (model: $\text{length} = \text{diet} + \text{sex}$) or wet weight (model: $\text{weight} = \text{diet} + \text{sex} + \text{large intestine length}$, mainly reflecting the effect of diet on the weight of content). As the time effect was found to be non-significant in rats fed for at least 16 days, we used two way models. In rats fed for at least 16 days, large intestine length was related both to sex and diet: the large intestine was longer when rats were fed one of the butyrate producing diets (RS, $p < 0.001$; FOS, $p = 0.01$). Although the caecum was the most affected, the colon was also longer, especially with the RS diet. Wet weights were linked to diet, sex, and large intestine length ($p < 0.001$). The weight of the large intestine content was greater with all fibre enriched diets (RS and FOS, $p < 0.001$; WB, $p = 0.08$, NS). The contents were not distributed in the same way. In the caecum, RS ($p = 0.002$) and FOS ($p = 0.04$) showed heavier contents than CD. In the colon, however, all fibre enriched diets showed heavier contents (males, 1.3-fold; females, 1.5-fold) than CD (WB, $p < 0.001$; RS, $p = 0.004$; FOS, $p = 0.07$, NS). The trophic effect of RS and FOS, which led to a macroscopically longer large intestine and larger caecum, was confirmed microscopically for the caecum (table 3). Neither the number of PCNA positive cells nor the height of the proliferative zone was diet related for any segment.

EFFECT OF DIET ON AZOXYMETHANE INDUCED ABERRANT CRYPT FOCI (TABLE 4)

As there was no effect of sex on fermentation, and colon morphometrics depend on sex, only male rats were used. Neither weight gain nor weight at sacrifice differed among diets. Counts by both observers were very similar (ACF, $r = 0.92$, $p < 0.001$; AC, $r = 0.95$, $p < 0.001$), and discrepancies concerned mainly single ACFs.

Table 4 Susceptibility to azoxymethane (AOM) of rats fed experimental diets (low fibre control diet (CD), starch free wheat bran enriched diet (WB), type III resistant starch enriched diet (RS), and short chain fructo-oligosaccharide enriched diet (FOS)) for 44 days

	CD	WB	RS	FOS
ACF count (mean (SEM))				
A	75.00 (3.24)	68.25 (12.97)	51.25 (10.14)	48.00 (6.74)
B	40.20 (2.73)	47.60 (7.98)	21.80 (7.81)	28.20 (6.72)
AC/ACF (mean (SEM))				
A	1.51 (0.02)	1.65 (0.04)	1.98 (0.18)	1.55 (0.10)
B	1.17 (0.10)	1.19 (0.06)	1.03 (0.07)	1.24 (0.11)

Two batches of six week old rats (A, n=16; B, n=20) were successively included in the study within a two week period. Rats were housed four per cage (randomly constituted blocks) until the beginning of the nutrition study, and then one per cage. They were first fed UAR A04 (pellets then powder), and then from D0 (10 weeks old) the powdered experimental diet allocated by randomisation. On D44, rats were injected twice subcutaneously, one week apart, with 15 mg/kg AOM. For each batch, rats were sacrificed one month later, at a rate of one or two blocks a day, that is, aberrant crypt foci (ACF) were scored 30–32 days after the first injection. The colon was separated longitudinally into two parts, only one was used for ACF count. The part kept for ACF count was divided into proximal and distal colon (according to the orientation of the mucosal folds), the latter being subdivided into two equal parts (upper and lower). ACF and individual crypts inside were scored, and then the total number of aberrant crypts (AC) were calculated. The AC/ACF ratio is indicative of crypt multiplicity.

Results were analysed using the ANOVA model $Y = \text{diet} + \text{batch}$, followed by Bonferroni's test. The batch effect was always significant ($p < 0.001$) whereas the diet effect was significant only for ACF.

Rats fed either butyrate producing fibre diets had a lower amount of ACF in the colon than those fed CD (RS, $p=0.022$; FOS, $p=0.043$). Crypt multiplicity (AC/ACF ratio) did not differ among diets.

Discussion

Our study has shown that for stable high butyrate production throughout the colon, both the substrate and time are critical factors in adult rats. At least two weeks are needed for adaptation to resistant starch,¹⁹ and four weeks to stabilise faecal weight and pH.²¹ In our study, butyrate increased unexpectedly over time with CD, maybe because starch, present in a larger amount than in the previous maintenance diet, partially escapes digestion in the small intestine.^{7 13 41} In contrast with other studies, WB was not a good long term butyrate producer. Entrapped starch may have been fermented to butyrate in these studies.^{20 42} In a four week experiment it was found that coarse (but not fine) wheat bran produced a high butyrate faecal concentration,²¹ with similar butyrate and propionate ratios, a classical fermentation profile for starch^{41 43 44} observed in our study. The transient butyrate production by WB could not be due to the fermentation of the fibre itself as the remaining indigestible components are readily fermentable, but rather produce acetate (hemicelluloses, mainly xy-lans)⁴³ or poorly fermentables (cellulose and lignin). The substrate(s) leading to butyrate production could be wheat bran proteins^{12 45} or partially protected available starch escaping digestion.⁴⁶ Endogenous substrates such as mucus or exfoliated cells could not be excluded⁶ as wheat bran particles that could abrade the mucosa were observed macroscopically as far as the proximal colon. Thus changes in WB fermentation may involve a host adaptation (increased secretion with time of proteolytic enzymes or α amylase, modifications of the mucosa) rather than a microflora adaptation. Although RS and FOS were ultimately high butyrate producers, differences were observed along the adaptive period. Adaptation of the microflora can be quantitative (bacterial

growth) and/or qualitative, involving bacteria cooperation^{41 47} and induction of specific activities.⁴⁸ Both RS and FOS ultimately induced bacterial growth, as indicated by caecal enlargement and increased weight.^{19 21 49} Differences between diets on D2 (our baseline) could only have been due to induction of specific activities of the microflora then present, and which were adapted to the previous maintenance diet. Fibres were fermented towards acetate (starch, and to a lower extent wheat bran), or propionate and lactate (FOS). This accumulation of lactate suggested that bacterial glycolysis to lactate was more rapid than its further metabolism to SCFA.^{43 50} The formation of propionate from lactate through the alternative acrylate pathway could explain its high concentration.^{41 47} From D16, butyrate concentration increased, as shown when rats were fed 5% cellulose+6% fructo-oligosaccharides for 14 days.⁴⁹ For RS, the typical fermentation pattern was apparent as early as D16, subsequent adaptation leading to a more complete breakdown of the fibre, until stabilisation on D44.

At the end of the adaptive period, we classified the diets as high butyrate (RS and FOS) and low butyrate (WB and CD) producers, the other SCFAs and the physicochemical characteristics of the fibres being different. As determination of SCFA was crucial, labelling of PCNA, a validated proliferation marker,^{15 51 52} was chosen to avoid the need to handle rats before sacrifice. It was important to control mucosal proliferation at the time chosen for induction as this could have modified the response to the carcinogen. The fact that no differences were observed between low and high fibre diets, or between low and high butyrate producing diets is not particularly surprising. When fibres stimulated proliferation, it was linked to SCFAs, especially butyrate, as also observed with colonic explants.^{15 53–55} However, as in our study, the proliferating cells remained in the lower 60% of the crypt.^{56 57} In contrast, in the context of high fat diets, which induce hyperproliferation with upward shift of the proliferative compartment, proliferation was decreased with starch.⁵⁸ However, as slow release pellets of sodium butyrate had no effect in the same context,⁵⁹ the decrease may be linked to the ability of starch to bind bile acids.⁶⁰ Most stimulatory effects of fibres and/or SCFAs were observed after short term ingestion of isolated fibres compared with fibre free diets, or during post-starvation recovery.¹⁷ Increasing butyrate concentration to more than 10 mmol/l in human caecal biopsies did not result in a further increase in cell labelling.¹⁵ Such a plateau could explain our results as the control diet produced butyrate. However, the duration of the experiment was probably the main reason. In other long term studies, no (or a very modest) increase in proliferation^{21 56–57 61} occurred with high fibre diets, and SCFAs did not correlate with various mucosal growth characteristics.^{21 56 62 63} In rats fed a high fibre diet (guar gum), proliferation of distal colonic mucosa returned to the level of the control diet after a transient increase over a

period of 9–21 days.⁵² The mucosa can also alter its growth characteristics by crypt duplication, increasing the number of crypts per unit length and total length in response to diet.^{16 17 63 64} Such an increase in length was apparent in our study with high butyrate producing diets, as well as deeper crypts in the caecum where fermentation was the most intensive.

In this study, both RS and FOS were protective against the first stages of carcinogenesis whereas WB was not. The protective effects had been related to a lower energy contribution, to dilution or adsorption of the carcinogen or to fermentation. Less weight gain, observed with high amounts of fibre, was avoided as was any adsorption of the carcinogen, because of the use of AOM.³¹ As all fibres led to a heavier content, any protective effect linked to dilution of the carcinogen would have been the same. As carcinogenesis was induced in homogeneous inbred rats, with stable colonic ecosystems and the same proliferation status, the remaining differences related to fibre and its modifications of luminal factors (for example, the microflora and its activity) and mucosal physiology (for example, the colonocyte phenotype). A short term study was preferred, focusing on the initiation and post-initiation stages alone, as AOM alters the microflora (reducing SCFAs) and colonocyte metabolism, and in the long term, interactions occur between the fibre source and the carcinogen.⁶⁵ Furthermore, in nutrition studies, ACF count was a predictor of tumour incidence whereas the size of a focus (multiplicity) that could increase in time was not.²⁸ In studies where resistant starch was not protective,^{28 66} type II instead of type III resistant starches were used, the period of adaptation to diets was very short, and rats were injected once a week for 10 or 20 weeks. Furthermore, as faecal starch content and output (when reported) were very high,²⁸ adaptation does not seem to have occurred in spite of the long course of the experiment, possibly because of modifications that the carcinogen induced in the colonic ecosystem. However, we cannot exclude the fact that starch may be protective in the initiation stage, due to butyrate, and promotive in later stages, due to propionate, as observed with propionate producing fibres.^{3 28 67 68} In the studies that showed a protective effect against colon tumours in rats, the source of butyrate was unprocessed wheat bran, generally in the context of high fat diets that involve the release of a large amount of biliary acids. The deleterious effect of the associated propionate could have been offset by other protective mechanisms such as trapping of luminal promoters.^{3 4 28}

Our study indicates that butyrate is associated with protection against the initial stages of colon carcinogenesis, regardless of the fibre source. Although fibre induced modifications of the microflora may be involved, it is more likely that butyrate itself is the effector. Indeed, results similar to ours were obtained with slow release pellets of sodium butyrate, that is, no effect on proliferation, and a lower count of ACF (albeit non-significant, may be due to the low number of rats) with no effect on

multiplicity.⁵⁹ That oral butyrate was not protective^{69 70} is probably due to its absorption before it reached the colon. The action of butyrate in situ is mediated by its capacity to modulate the colonocyte phenotype.^{9 15 71 72} However, it is likely that the colonic mucosa would not stabilise, thus colonocytes were of a given susceptibility to carcinogenesis, until the colonic ecosystem itself became stable. Hence long term adaptation to a diet may not only concern the microflora. The colonocyte is involved in complex tissue homeostatic inter-relationships with other mucosal cells, including immune cells. We have previously shown that butyrate modulates the phenotype and immunogenicity of rat colon cancer cells, allowing a specific immune response in the context of immunotherapy against intraperitoneal carcinomatosis.^{71 72} One possible mechanism for the protective effect of butyrate producing fibres could be stimulation of colonocyte immunogenicity as elevated immune cytotoxicity may contribute to inhibition of AOM induced ACF in rats.⁷³ This hypothesis is supported by our results in another colon cancer model.⁷⁴

From a preventive viewpoint, butyrate producing fibres seem very promising, although the nature and processing of the fibre need to be carefully controlled to provide sufficient production throughout the colon. Naturally occurring fibres such as wheat bran could be selected as several of their compounds provide synergic protective effects. However, as the composition and processing of this fibre would interfere with its protective capacities, it may be preferable to use combinations of chemically well defined carbohydrates. This study may help define the carbohydrates of interest.

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