

## Butyrate is only one of several growth inhibitors produced during gut flora-mediated fermentation of dietary fibre sources

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Dietary fibre sources are fermented by the gut flora to yield short-chain fatty acids (SCFA) together with degraded phytochemicals and plant nutrients. Butyrate, a major SCFA, is potentially chemoprotective by suppressing the growth of tumour cells and enhancing their differentiation. Conversely, it could lead to a positive selection pressure for transformed cells by inducing glutathione S-transferases (GST) and enhancing chemoresistance. Virtually nothing is known about how butyrate's activities are affected by other fermentation products. To investigate such interactions, a variety of dietary fibre sources was fermented with human faecal slurries *in vitro*, analysed for SCFA, and corresponding SCFA mixtures were prepared. HT29 colon tumour cells were treated for 72 h with individual SCFA or complex samples. The growth of cells, GST activity, and chemoresistance towards 4-hydroxynonenal were determined. Fermentation products inhibited cell growth more than the corresponding SCFA mixtures, and the SCFA mixtures were more active than butyrate, probably due to phytoprotectants and to propionate, respectively, which also inhibit cell growth. Only butyrate induced GST, whereas chemoresistance was caused by selected SCFA mixtures, but not by all corresponding fermentation samples. In summary, fermentation supernatant fractions contain compounds that: (1) enhance the anti-proliferative properties of butyrate (propionate, phytochemical fraction); (2) do not alter its capacity to induce GST; (3) prevent chemoresistance in tumour cells. It can be concluded that fermented dietary fibre sources are more potent inhibitors of tumour cell growth than butyrate alone, and also contain ingredients which counteract the undesired positive selection pressures that higher concentrations of butyrate induce in tumour cells.

### Short-chain fatty acids: Complex gut fermentation products: Genotoxicity: 4-Hydroxynonenal

There is a continuing need to assess how plant foods contribute to reducing risks of colorectal cancer development and to gain a better understanding of putative mechanisms (Harris & Ferguson, 1993; Hill, 1995). Dietary fibre increases stool bulk and speeds the transit time of faeces (Baghurst *et al.* 1996). It also modulates the metabolism of food-borne carcinogens and scavenges reactive intermediates (Ryden & Robertson, 1995; Kestell *et al.* 1999). A further important property is the increased generation of butyrate from dietary fibre by the gut flora (Cummings,

1981). Butyrate is physiologically relevant to the colonic epithelium which it serves as a principal energy source (Silvester *et al.* 1998). Interest in its role as a possible protective agent has arisen from its anti-proliferative effects on cells *in vitro* (Kruh, 1982), including colon tumour cell lines (Gamet *et al.* 1992; Hague & Paraskeva, 1995). It also protects against H<sub>2</sub>O<sub>2</sub>-induced genetic damage in primary colon cells (Abrahamse *et al.* 1999) and in colon tumour cell lines (Rosignoli *et al.* 2001). Moreover, at low concentrations sodium butyrate induces apoptosis in

**Abbreviations:** ABTS, 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonic acid); AP, alkaline phosphatase; EC<sub>50</sub>, effective concentration of a test sample resulting in a 50% reduction of cell number under the specified cell culture and treatment conditions; GSH, glutathione; GST, glutathione S-transferase; HNE, 4-hydroxynonenal; SCFA, short-chain fatty acid; TEAC, Trolox-equivalent antioxidant capacity.

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human colon carcinoma cell lines (Hague & Paraskeva, 1995). Growth inhibition, anti-genotoxicity and apoptosis contribute to anti-cancer activities by inhibiting initiation and progression or by removing initiated cells from the tissue (Wattenberg, 1992; Johnson *et al.* 1994). In extension of this theory, dietary fibre is considered to exert part of its protective effects via butyrate formation in the gut lumen (Perrin *et al.* 2001; McIntyre *et al.* 1993). However, this theory has several flaws, the major one being that there is virtually no evidence available to support the assumption that these mechanisms also occur *in vivo* (Lupton, 1995). Moreover, little is known about how butyrate affects human colon cells in conjunction with the other fermentation products, which are formed simultaneously. The cellular effects of physiological combinations of short-chain fatty acids (SCFA) have been little investigated, even for the most straightforward parameter of growth inhibition; neither have the additional effects of other plant ingredients and nutrients been assessed in this context. The other SCFA are mainly acetate and propionate, as well as the minor components valerate, hexanoate and branched SCFA (isobutyrate and isovalerate) (Cummings, 1981; Cummings *et al.* 1987). It has already been shown that the relative and absolute molar concentrations of SCFA produced during fermentation are highly different for different fibres, thus providing one explanation as to why different fibres have different impacts in the gut (Ferguson *et al.* 2001). The ingestion of plant products results in the presence of a wide variety of phytochemicals and nutrients in the gut. Although very little is known about the quality and quantity of these compounds, experimental studies with individual components or with plant extracts suggest that they could substantially contribute to anti-cancer effects and perhaps provide another explanation for the different activities of different fibre sources (World Cancer Research Fund and American Institute for Cancer Research, 1997). Fermentation products can be generated *in vitro* using anaerobic protocols that simulate the conditions of the human gut lumen. This provides an experimental approach to compare the biological activities of different dietary fibre sources and of their fermentation products (Wang & Gibson, 1993). The aim of the present work was to use such methods to study the effects of various fermented fibre sources and compare their anti-tumour activities using physiologically relevant concentrations of SCFA in HT29 colon tumour cells.

Various vegetable samples and other dietary fibre sources were fermented with human gut flora *in vitro* and the SCFA product was determined. These *in vitro* fermentation samples, as well as corresponding mixtures, containing the three major pure SCFA in identical concentrations and molar ratios, were investigated for the kinetics of inhibiting the growth of colon cells in culture. The growth-inhibitory properties of the mixtures were then compared with the activities of butyrate, acetate, propionate, lactate, and valerate. The sensitivities of HT29 parent cells and the differentiated sub-clone, HT29 clone 19A, were also assessed (Augeron & Laboisse, 1984). Additionally, the present study measured how the samples affected the activity of glutathione S-transferase (GST), glutathione (GSH) concentration, and the activity of

alkaline phosphatase (AP) as markers of differentiation. The induction of GST is connected to increased inactivation of the cancer risk factor, 4-hydroxynonenal (HNE). This physiologically formed product of lipid peroxidation is highly genotoxic, an excellent substrate for GST, and its genotoxicity can be substantially decreased by pre-treating HT29 cells with butyrate (Ebert *et al.* 2001). The present study also investigated whether the complex samples also mediate chemoresistance in the tumour cells. The results of the study were expected to enhance our knowledge of the relative suppressing activities caused by butyrate in comparison with SCFA mixtures and by the SCFA mixtures in comparison with the complete fermentation sample.

## Materials and methods

### *Test chemicals and miscellaneous other reagents*

Na-butyrate (CAS no. 156-54-7), Na-propionate, Na-acetate, 1-chloro-2,4-dinitrobenzene (CAS no. 97-00-7), and GSH (reduced) (CAS no. 70-18-8) were obtained from Merck kJ, Darmstadt and Merck-Schuchardt, Hohenbrunn, Germany, respectively. HNE (CAS no. 75899-68-2) was from Calbiochem-Novabiochem GmbH, Bad Soden, Germany. p-Nitrophenol and p-nitrophenolphosphate were obtained from ICN Biochemicals, Eschwege, Germany. All these chemicals were 99% pure. SCFA were dissolved in Dulbecco's modified Eagle's medium (Life Technologies, Karlsruhe, Germany) for cell incubation experiments. All chemicals were of the highest commercially available grade of purity.

### *Food supplements*

The investigations were performed with fermentation samples of different potential food supplements of plant origin (Kampffmeyer Food Service GmbH, Hamburg, Germany). Their major functional ingredients as specified for technological applications are summarised in Table 1. All samples were portioned and stored in sealed containers under inert gas at  $-20^{\circ}\text{C}$  until preparation.

### *Determination of fibre fractions*

The method for the determination of total dietary fibre requires the enzymic digestion of protein and non-resistant starch, followed by the precipitation of soluble fibre with alcohol and weighing (Association of Official Analytical Chemists International, 1992). The method of Van Soest *et al.* (1991) was used to determine neutral-detergent fibre and acid-detergent fibre in different food supplements by using the Fibertec™ 1020 System M6 (FOSS Tecator, Hillerød, Denmark).

### *Analytical determinations*

SCFA were determined in the fermentation supernatant fractions of dietary fibre. After extraction with ice-cold water, the gas-chromatographic measurements (Shimadzu GC 17A; Shimadzu, Kyoto, Japan) were done using a

**Table 1.** Characteristics of dietary fibres  
(Mean values of duplicate determinations\*)

| Fermented from             | Marker compounds                   | Total dietary fibre† (%) | Neutral-detergent fraction‡ (%) | Acid-detergent fraction§ (%) | Total phenols (mg GAE/g) | TEAC before fermentation (mmol/l per g) | TEAC lipid before fermentation (mmol/l per g) | TEAC after fermentation (mmol/l per g) |
|----------------------------|------------------------------------|--------------------------|---------------------------------|------------------------------|--------------------------|-----------------------------------------|-----------------------------------------------|----------------------------------------|
| Control or blank           | 0                                  | /                        | /                               | /                            | /                        | /                                       | /                                             | /                                      |
| Linseed (defatted)         | About 3.3% di-glucosylated lignans | 45.8                     | 30.3                            | 13.7                         | 2.22                     | n.d.                                    | 0.0003                                        | n.d.                                   |
| Vegetable 3 (watercress)   | About 0.12% glucosinolates         | 43.7                     | 32.0                            | 27.8                         | 4.34                     | 0.003                                   | 0.003                                         | n.d.                                   |
| Vegetable 2 (kale)         | About 0.08% lutein and zeaxanthin  | 5.2                      | 0.3                             | 0.6                          | 20.97                    | 0.004                                   | 0.040                                         | 3.86±0.03                              |
| Tomato                     | Lycopene, about 6% carotenoids¶    | 7.0                      | n.d.                            | n.d.                         | 1.68                     | 0.003                                   | 0.107                                         | n.d.                                   |
| Soya (soya flour)          | About 3% isoflavonoids             | 14.2                     | 13.1                            | 3.5                          | 3.4                      | 0.003                                   | 0.116                                         | 0.16±0.00                              |
| Inulin (chicory)           | > 90% Fructo-oligosaccharides      | 15.7                     | n.d.                            | n.d.                         | /                        | n.d.                                    | 0.008                                         | n.d.                                   |
| Wheat                      | 4 ppm Se, S**                      | 13.7                     | 11.7                            | 2.9                          | 0.47                     | n.d.                                    | 0.108                                         | n.d.                                   |
| Yeast (cell-wall fraction) | About 70% glucans                  | 76.8                     | n.d.                            | n.d.                         | 0.26                     | n.d.                                    | 0.019                                         | n.d.                                   |

GAE, gallic-acid equivalents; TEAC, Trolox-equivalent antioxidant capacity; /, not determined; n.d., not detectable; ppm, parts per million.

\* With extremes not exceeding 10% of the mean.

† Total dietary fibre before fermentation was determined according to van Soest (Paikin *et al.* 1988).

‡ Neutral-detergent fibre (hemi-cellulose, cellulose and lignin).

§ Acid-detergent fibre (cellulose and lignin).

|| The detected glucosinolates were progoitrin (0.41 µmol/100 g), gluconapoleiferin (32.5 µmol/100 g), gluconapin (0.11 µmol/100 g), 4-hydroxyglucobrassicin (n.d.), glucobrassicinapin (3.52 µmol/100 g), total glucosinolates (36.54 µmol/100 g).

¶ Lycopene (3848 mg/100 g), total β-carotene (195 mg/100 g), e-β-carotene (90 mg/100 g).

\*\* Se (397 µg/100 g), S (0.18 g/100 g).

15 m FFAP-column and a temperature program (start temperature 130°C, increase 35°C/min and final temperature 170°C; Kiessling *et al.* 2002).

Glucosinolates were detected by HPLC (LC-6 Analyser, Seperon SGX-C<sub>18</sub>-Column; Shimadzu, Kyoto, Japan) with sinigrin as an internal standard (Anonymous, 1990).

Se content was analysed by graphite furnace atomic absorption spectrometry, and S content by using an elemental analyser (Elementar Analysensysteme, Hanau, Germany).

Carotenoids were analysed by saponifying samples of about 0.2 g using 40% (w/v) potassium hydroxide in methanol and extracting with hexane until the residue was colourless. The upper layer was analysed with C<sub>18</sub> HPLC on a VYDAC 201TP54 column with diode array detection (Böhm, 1999).

Total phenolics were determined by using the Folin-Ciocalteu method as described by Schlesier *et al.* (2002).

#### Antioxidative activity

Antioxidant activity was determined in the hydrophilic extract as well as in the lipophilic extract using the Trolox-equivalent antioxidant capacity (TEAC) assay (TEAC I and TEAC 2, respectively) (Schlesier *et al.* 2002). The hydrophilic test is based on the oxidation of 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonic acid (ABTS) in the presence of H<sub>2</sub>O<sub>2</sub> and metmyoglobin to the radical cation ABTS<sup>•+</sup> (blue-green colour), which is photometrically measured at 734 nm. Dependent on the concentration of radical-trapping substances, oxidation is delayed. For the lipophilic TEAC assay the ABTS<sup>•+</sup> radical cation was prepared by filtering a solution of ABTS through manganese dioxide powder. The ABTS<sup>•+</sup> solution and the solution with antioxidants were vortexed for 30 s in reaction tubes, which were then centrifuged for 60 s at 10 000 rpm. The absorbance (734 nm) of the lower phase was taken exactly 2 min after the initiation of mixing. By using the decrease in absorbance, the antioxidant activity can be calculated.

#### Fermentation of dietary fibre sources

All fermentations were conducted *in vitro* under anaerobic conditions (86% N<sub>2</sub>, 10% CO<sub>2</sub>, 4% H<sub>2</sub> at 37°C, by vol.). Fresh human faeces were used as a bacterial source and they were fermented with the reconstituted food powders. The fibre contents presented in Table 1 were used to determine the amount of freeze-dried food sample needed to provide 20 g fibre/l. The samples were then reconstituted with anaerobic potassium phosphate buffer (0.1 M, pH 7.0) to provide 20 g fibre/l. Fresh faeces collected from two healthy human volunteers was collected as the source of gut floras. The fermentations were carried out at the same time using the same pooled faecal suspension. The samples were immediately weighed and combined in a large homogenising bag. Pre-warmed potassium phosphate buffer was added (5:1, v/w) and the mixture was homogenised thoroughly in a Stomacher 400 (a unique instrument for homogenising in which the sample is effectively blended within a special disposable bag; Seward, Thetford,

Norfolk, UK). Samples (20 ml) of the faecal homogenate were measured into 50 ml centrifuge tubes in an anaerobic cabinet. Then 20 ml of each food sample was added to the separate tubes to give a final fibre content of 10 g/l and a faecal suspension of 10% (w/v) as recommended by Barry *et al.* (1995). Potassium phosphate buffer was added to one tube, as a negative control, and 40 ml phosphate buffer was included as a blank. Each tube was vigorously shaken to mix well. The fermentations were performed for 24 h. Each tube was manually mixed periodically throughout the procedure (every hour for the first 8 h and every hour for the final 8 h). Placing the suspensions on ice then stopped the fermentations. Each tube was centrifuged at 6000 g at 4°C for 30 min and the respective supernatant fractions were then portioned into appropriately labelled tubes and stored at -20°C. The samples were sterilised by filtration (pore size 0.22 µm) before they were added to the cell culture medium.

#### *Cell lines and culture conditions*

The human colon carcinoma cell line HT29 used in the present study was established by J. Fogh and Trempe in 1964 and originated from an adenoma colon tissue. It was obtained from the American Tissue Culture Collection (Rockville, MD, USA). The subclone 19A is a permanently differentiated clone derived from parent cells treated with butyrate (Augeron & Labosse, 1984). Both cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and penicillin (50 IU/ml)-streptomycin (50 µg/ml) in a humidified 5% (v/v) CO<sub>2</sub> incubator at 37°C. The cell lines grew strictly adherent. Under the given laboratory conditions parent cells doubled their number within 22 h and clone 19A cells within 24 h. Passages 35-45 were used.

#### *Determination of cell growth*

The growth and survival of colon cells were determined in ninety-six-well microtitre plates. At 48 h after seeding, cells were treated with SCFA (0.1-100 mM) in the culture medium. Fermented dietary fibres and the corresponding SCFA mixtures were analysed in parallel by adding different volumes, yielding final concentrations from 2.5 to 25% (v/v). Cells were treated for 72 h and surviving cells were measured. DNA was isolated by fixing and permeabilising the cells with methanol for 5 min, followed by the addition of 4',6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich, Deisenhofen, Germany). After 30 min, DNA content, as a reflection of the remaining cells, was detected by fluorimetric analysis with excitation and emission at 360 and 465 nm. The mean values (three determinations per experiment, three to four experiments) were recorded for the final evaluation.

#### *Modulation of 4-hydroxynonenal-induced DNA damage*

Each test consisted of three culture flasks (each with  $2 \times 10^6$  cells/ml): one with cells treated for 72 h with SCFA, one with fermentation supernatant fractions, and the third with untreated cells. The cells were subsequently

incubated with 150 µM HNE for 30 min. Viabilities were determined with trypan blue exclusion and the remaining cells were mixed with low-melting agarose and distributed onto microscopic slides. Further steps were carried out as described before (Singh *et al.* 1988; Ebert *et al.* 2001). In short, the slides were lysed for 60 min at 4°C and subjected to alkaline conditions for 20 min. Electrophoresis was carried out at 25 V and 300 mA for 20 min, after which the slides were neutralised and stained with ethidium bromide (Sigma, Deisenhofen, Germany). Comet images, revealing more or less damaged DNA (Singh *et al.* 1988) were quantified using the image analysis system of Perceptive Instruments (Halstead, Essex, UK). Fifty DNA spots were evaluated per slide. The mean values of tail intensity (the percentage of fluorescence in the comet tail) from three slides per experiment were calculated and the means of at least three independently reproduced experiments are the basis for the data presented in the graphs.

#### *Determination of glutathione S-transferase and alkaline phosphatase activities, total protein and glutathione*

For this the cytosol was prepared as described before (Ebert *et al.* 2001). The suspension was then homogenised for 1 min at approximately 30 W with ultrasound (Bandelin Electronics, Berlin, Germany) (Pool-Zobel *et al.* 1998; Treptow-van Lishaut *et al.* 1999). Following ultra-centrifugation (105 000 g, 75 min, 4°C), the supernatant fraction was sampled and frozen at -80°C until required for further work. This included the determination of total GST activity spectrophotometrically at 340 nm using 1 mM-1-chloro-2,4-dinitrobenzene and 1 mM-GSH as substrates and a temperature of 30°C (Habig *et al.* 1974). AP activity was measured by the hydrolysis of p-nitrophenylphosphate (5 mM) using 50 µl cytosol and 150 µl tri(hydroxymethyl)-aminomethane-HCl buffer (50 mM, pH 10) at 37°C (Bowers & McComb, 1966). p-Nitrophenol (0-200 µM) was used as the standard curve. Enzyme activity was expressed in units, each unit being equivalent to the number of µmol p-nitrophenol liberated in 1 min measured at 400 nm. Total protein content was measured using the method of Bradford with bovine serum albumin as the standard protein (Bradford, 1976). Intracellular GSH levels were determined using a colorimetric assay (Glutathione Assay Kit; Calbiochem-Novabiochem GmbH, Bad Soden, Germany). All values were based on the number of cells as has been described previously (Treptow-van Lishaut *et al.* 1999) and total protein content, respectively. Three parallel determinations were performed for each of these assays. The mean values of at least three independent experiments are plotted in the graphs.

#### *Statistical evaluations*

Means were calculated from at least three independently reproduced experiments (each data point obtained in triplicate per experiment). Medium-coarse cubic spline curves with forty segments were calculated as standard curves. The effective concentrations of test samples resulting in a 50% reduction of cell number under the specified cell culture and treatment conditions (EC<sub>50</sub>) were calculated

from non-linear regression and expressed as Log (EC<sub>50</sub>); best-curve fits were generated from top (constant at 100) to bottom (variable or = 0) with GraphPad Prism (Graph-Pad Software, Inc., San Diego, CA, USA) unless otherwise specified. Differences were calculated by one- or two-way ANOVA, including post hoc tests appropriate for the individual data sets, as is indicated in the Figs. and Tables.

## Results

### *Properties of dietary fibre sources*

A large group of plant food samples are being developed and characterised as potentially beneficial ingredients of new functional foods, in particular for baked goods. They were chosen on the basis of suitability in terms of taste, colour, baking properties, costs and other industrial requirements, but the major selection criterion for the present study was that they should contain considerable amounts of functional ingredients (a range of phytoprotectants and dietary fibres). All samples were first characterised for their content of key ingredients (marker compounds) and for their solubility in water or in organic solvents. The water-soluble samples (containing green-tea catechins and anthocyanins and anthocyanidins) were investigated separately (Matuschek *et al.* 2001; Glei *et al.* 2003). Samples which were mainly soluble in organic solvents (carotene- and lycopene-rich plant products) were not further investigated in the present study, since previous work had shown that the marker compounds either did not protect colon cells from oxidative stress or that the appropriate solvents were too toxic for the cell culture conditions (Vosseler, 2000; Glei *et al.* 2002). The remaining samples (Table 1), which were largely insoluble in water, were to be subjected to fermentation. They contained a heterogeneous range of major marker compounds and several different types of dietary fibre, each of which was characterised in more detail. Total dietary fibre was isolated, which includes cell-wall or cell-skeleton material, and also 'detergent fibre', defined as the residue of plant cells after fractionation with detergent solutions. Two fibre mixtures were obtained: (1) an acid-detergent solution with liberated acid-'detergent fibre', consisting of the total cellulose and lignin; (2) a neutral-detergent solution with neutral-'detergent fibre', consisting of the hemi-cellulose, cellulose and lignin. Some of the dietary fibre sources contained as marker compounds (isoflavonoids, lignans) have already been characterised for basic activities *in vitro* and *in vivo* (Pool-Zobel *et al.* 2000) or may be subject to biological analysis at a later time (glucosinolates, Se). The focus of the present study, however, was to study the consequence of the biological activities of the non-soluble samples after metabolism by the gut flora. Therefore the complete samples were subjected to *in vitro* fermentation with human faecal slurries. This resulted in a complete loss of antioxidative activities for some of the samples such as vegetable 3 or wheat (Table 1). In contrast, vegetable 2 had a significantly higher antioxidant potential after fermentation, possibly due to the liberation of antioxidant aglycones from plant complexes. Tomato, soya and vegetable 2 all had a higher antioxidant capacity in the lipophilic extract than in the water extracts, which could be due to the presence of lipophilic antioxidants, such as carotenoids.

### *Components of fermented samples*

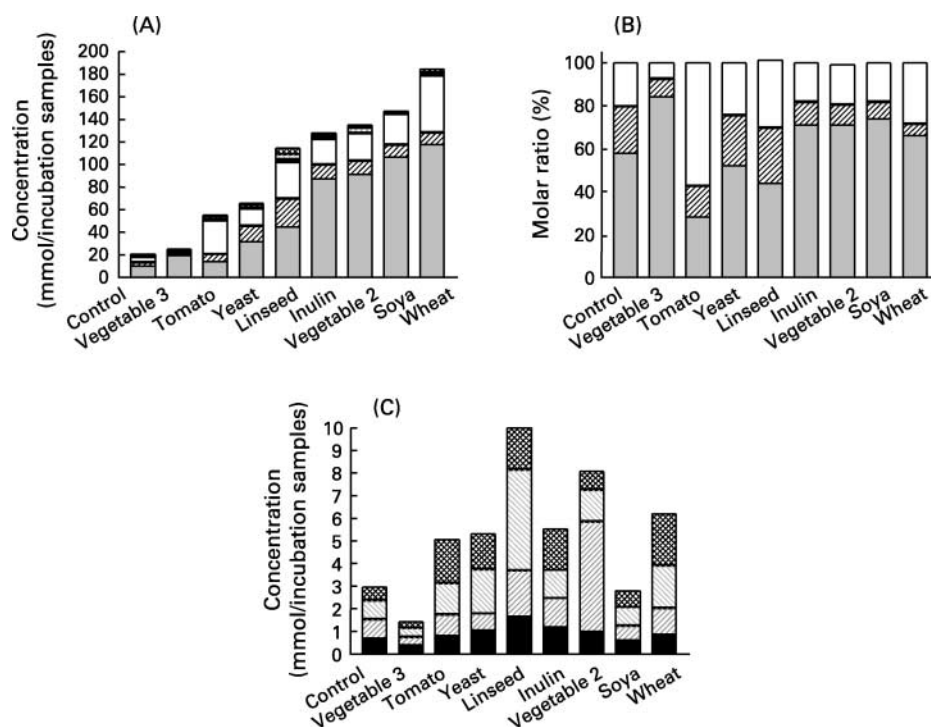
The fermentation supernatant fractions were further characterised for their content of SCFA. Fig. 1 (A) shows that there are considerable differences in the yields of SCFA, with a wide concentration range of about 25 to 180 mmol per 10 g fibre in 1 litre fermentation sample. The major product is acetate (20 to 118 mM), followed by butyrate (1.6 to 50 mM) and propionate (2.2 to 26 mM). The highest levels of valerate and capronate were produced from linseed flour (approximately 4.5 mM) followed by wheat, which yielded approximately 2 mmol/l of each (Fig. 1 (C)). Branched SCFA, which are a reflection of protein fermentation, were produced mostly from linseed and vegetable 2, with 4.5 and 2.1 mM-isovalerate, respectively, whereas isobutyrate concentrations were 2-fold lower. The latter was present at maximally 1.7 mM in the linseed-fermentation supernatant fractions. Fig. 1 (B), which shows the relative molar ratios of the three most abundant SCFA, reveals that the sample prepared from tomato had the highest relative proportions of butyrate, followed by linseed and then by wheat. On the basis of the original fibre contents of the food supplements (Fig. 2), it is apparent that the quality of the ingredients, not so much the total fibre quantity, is responsible for the absolute yield and molar ratios of SCFA in the fermentation samples. For example, Fig. 2 (A) shows that yeast and vegetable 3 have high levels of fibre, but produce only less than 100 mmol SCFA/l during fermentation (Fig. 1(A)). Also, the fibre content (acid- and neutral-'detergent fibre' fractions) is inversely related to the yield of total SCFA for the four samples vegetable 3, linseed, soya and wheat (Fig. 2 (B) and (C)). Vegetable 2, which ranks third highest in respect to SCFA yield, has a low fibre content, but particularly high levels of polyphenols (Fig. 2 (D)).

### *Modulation of cell growth by individual short-chain fatty acids*

The growth of the cells was efficiently retarded in HT29 parent cells by all SCFA. The Log EC<sub>50</sub> values after 72 h treatment were 46, 21, 11, 3, and 2 mM for acetate, lactate, valerate, butyrate and propionate, respectively. The corresponding values for the HT29 clone 19A cells were 26, 18, 11, 3 and 3 mM, respectively. Significant differences of cell growth between HT29 parent and clone cells were apparent only for acetate and lactate.

### *Modulation of cell growth by complex samples*

The cellular effects of the fermentation supernatant fractions and of mixtures of SCFA were next investigated, prepared to mimic the concentrations observed in the original fermentation samples. Fig. 3 (A) shows that quite different dose-response curves were observed for the individual fermentation samples. Some were inhibitory already at concentrations of 2.5–5% (wheat), whereas the control and vegetable 3 had no marked effects on cell growth up to a concentration of 15% (v/v). The synthetic SCFA mixtures were less effective (Fig. 3 (C)), but the



**Fig. 1.** (A), Absolute concentrations of major short-chain fatty acids (SCFA) produced after fermenting the samples with human gut flora *in vitro*; (B), relative molar concentrations of the major SCFA (acetate, propionate, butyrate); (C) absolute concentrations of minor SCFA produced after fermenting the samples with human gut flora *in vitro*. Samples are sorted according to increasing SCFA content. (▨), Acetate; (▩), propionate; (□), butyrate; (■), isobutyrate; (▧), isovalerate; (▨), n-valerate; (▩), n-caproate.

general profile of response was similar; the synthetic mixture mimicking the SCFA profile of wheat, for example, was most toxic, whereas cell survival was less affected by the control and by vegetable 3 up to concentrations of 15–20% (v/v). When comparing the response curves obtained after plotting the butyrate concentrations (Fig. 3 (B) and (D)), a completely different picture is apparent and the results for most samples are very homogeneous. With the exception of tomato fibre, vegetable 3 and the control, containing the lowest SCFA levels, all curves of the fermentation samples shown in Fig. 3 (B) follow a similar pattern of response. The curves for the corresponding SCFA mixtures (also plotted on the basis of their butyrate concentrations) are again similar (Fig. 3 (D)), although the samples have a slightly lower impact than the complete fermentation supernatant fractions.

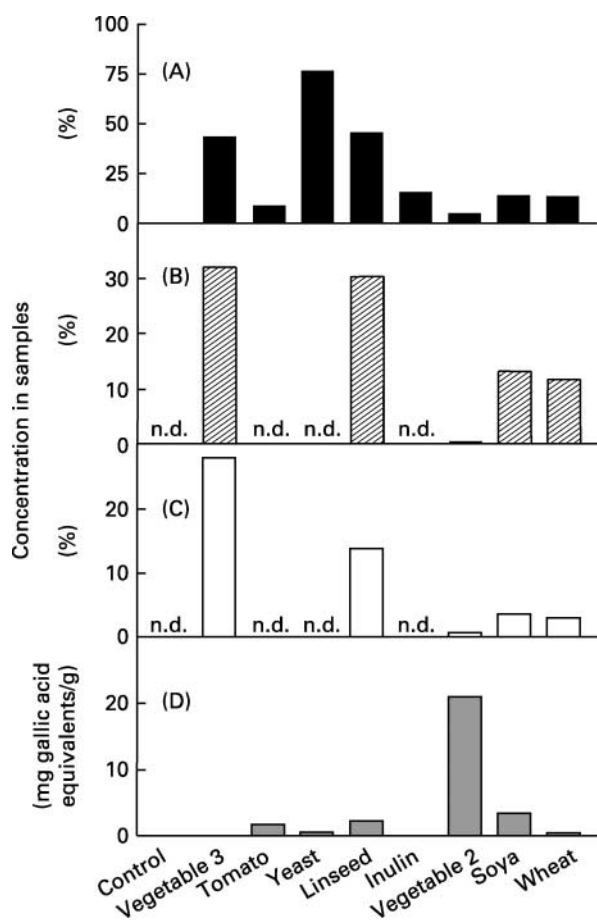
From these data and from an identical set of data obtained for the HT29 clone cells (data not shown), the  $EC_{50}$  of each sample was calculated and used for further comparative analyses. Table 2 compares the data for both cell lines and for the complete fermentation supernatant fractions *v.* their corresponding SCFA mixtures. In most cases, lower amounts of fermentation samples than SCFA mixtures are needed to achieve the inhibitory levels. These differences between complete fermentation supernatant fractions and their corresponding SCFA mixtures are statistically significant for all samples in HT29 clone cells and for all except for yeast, inulin and soya in HT29 parent cells. When comparing the two cell lines, it is apparent that clone cells are sometimes more sensitive towards the complex samples than parent cells. This is significant for the fermentation supernatant fractions

from yeast, inulin, and vegetable 2, as well as for the SCFA mixtures composed according to tomato, vegetable 2, and soya fermentations.

The values for the relative growth inhibitory concentrations by the fermentation samples ranged from 2.8 to over 50% in the parent strain. Equipotent amounts of supernatants contained similar amounts of butyrate and propionate, but differed in their acetate concentrations (Fig. 4). The estimated growth inhibitory amounts of fermentation samples from the control and from vegetable 3 were multifold higher and not plotted to enable a clearer presentation of the interactions of the other effective samples. Accordingly, the total amount of butyrate and propionate in the inhibitory volume is a direct mirror of the sample volume needed to achieve the inhibition of cell growth, as shown in Fig. 5. For the example of linseed, the original SCFA mixture with three components was compared with another SCFA mixture containing all seven SCFA (for example, additionally the four minor components shown in Fig. 1). The complete fermentation sample again was more active in HT29 parent cells than both SCFA mixtures, which were identical inhibitors and had overlapping growth curves (results not shown). Thus any additional effect of valerate and other SCFA, when present in the physiologically relevant concentrations shown in Fig. 1, is marginal.

#### *Combination effects of butyrate and other short-chain fatty acids*

The relative impact of butyrate is highly dependent on the additional factors present. When investigated as a sole



**Fig. 2.** Concentrations of total dietary fibres (A), neutral-detergent fibres (hemi-cellulose, cellulose and lignin; B), acid-detergent fibres (cellulose and lignin; C) and total polyphenols (as gallic-acid equivalents; D). n.d., Not detectable in the sample.

compound, only dissolved in medium, the inhibitory concentrations are much higher than when investigated in any of the combinations, for example together with either one, two or many SCFA. These relative potencies are similar in both cell lines (Fig. 5).

#### *Modulation of glutathione, glutathione S-transferase and alkaline phosphatase activities*

Propionate concentrations of 2.5 to 10 mM fully inhibit butyrate's inducing effects on GSH levels (11.2 (SD 1.2) and 12.5 (SD 2.2) nmol GSH/10<sup>6</sup> HT29 clone 19A and HT29 cells, respectively), without, however, leading to depletion (Fig. 6). Acetate (15–60 mM) had no effect on butyrate-induced GSH levels.

The concentrations used to determine the effects of selected fermentation products in modulating the activities of GST and AP shown in Table 3 are approximately 75 % of the EC<sub>50</sub>. However, EC<sub>50</sub> concentrations did not induce GST or AP activities (results not shown). Also none of the complex samples were able to induce GST or AP activities, maybe on account of low butyrate contents. The butyrate concentrations in the reconstituted SCFA mixtures and in the complex fermentation samples at equipotent growth inhibitory concentrations are 2.17 and 1.40 mM for wheat,

1.77 and 1.40 mM for soya, 2.06 and 1.26 mM for vegetable 2, 1.28 and 1.62 mM for inulin, 1.94 and 1.01 mM for linseed, 1.20 and 1.38 mM for yeast, and 0.42 and 1.87 mM in the blank. Butyrate induces GST activity 1.4-fold and AP activity 4-fold at 4.0 mM, which corresponds to 125 % of its EC<sub>50</sub>, a concentration at which only few cells survive or most are clearly impaired.

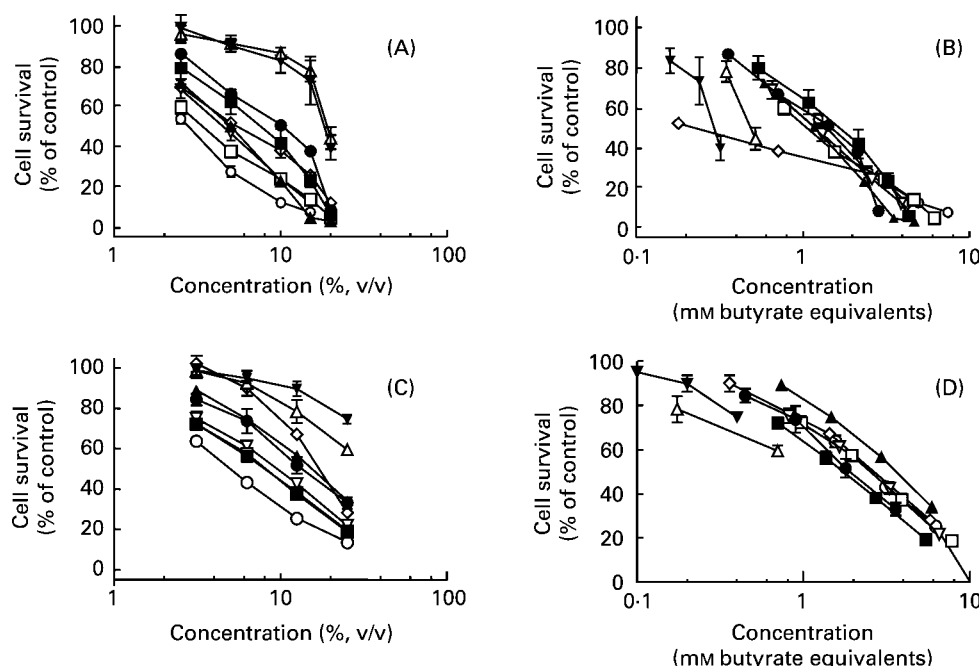
#### *Induction of chemoresistance*

Finally, the consequences of the inducible GST–GSH system were looked at, by first pre-treating the cells for 72 h with the fermentation samples and corresponding SCFA mixtures. Cells pre-incubated with medium were used as the untreated control. Subsequent to this first treatment, the cells were exposed to HNE for 30 min, and then assessed for HNE-induced DNA damage. Fig. 7 shows that HNE is genotoxic in non-pre-treated cells. However, after pre-treatment with butyrate and with physiological SCFA mixtures (made up according to the concentrations also found in the fermentation supernatant fractions of the respective dietary fibres), the genotoxicity of HNE was abolished in all four investigated samples. The actual fermentation samples prepared after incubating the complete mixture without substrate (control), or with inulin and wheat as a substrate, in contrast, did not diminish HNE-mediated genotoxicity. Only the fermentation sample of soya was as effective as butyrate and its SCFA for protecting the tumour cells from HNE genotoxicity.

#### **Discussion**

The human gut microflora has significant impacts on the host for health and disease; some flora producing toxins, whilst others produce SCFA, especially butyrate. Several findings point to a central role of butyrate as an important protective factor; it serves as an energy source (Roediger, 1989), protects from H<sub>2</sub>O<sub>2</sub>-mediated DNA damage (Pool-Zobel *et al.* 1996; Abrahamse *et al.* 1999; Rosignoli *et al.* 2001), and acts as a suppressing agent *in vitro* (Wattenberg 1992; Hague *et al.* 1993). Interestingly, butyrate often has opposite effects on tumour cells *in vitro* and on non-transformed cells *in vivo*, a phenomenon that is commonly referred to as the 'butyrate paradox'. The reasons for this could be the different stages of cell transformation (Hague & Paraskeva 1995), relative states of cellular activation (Gibson *et al.* 1999), or differences due to exposing cells removed from their natural environment (Lupton, 1995). Since human *in vivo* data on the suppressing activities by butyrate are not available, assumptions on anti-cancer potential by this compound are mainly derived from animal carcinogenesis studies, which have shown that luminal butyrate concentrations are inversely correlated with the development of chemically induced colon tumours (McIntyre *et al.* 1993; Compher *et al.* 1999; McIntosh *et al.* 2001).

The hypothesis, however, has a major drawback, since it is a matter of debate whether dietary fibre actually does play an important role in human cancer prevention (Schatzkin *et al.* 1995). For example, some large and well-conducted epidemiological studies do not support



**Fig. 3.** Modulation of cell growth (HT29 parent cells) after incubation of samples with fermentation supernatant fractions (A, B) or with corresponding short-chain fatty acid mixtures (C, D). Data are expressed on the basis of amount of sample added to achieve the respective (% v/v) concentrations (A, C) or on the basis of the molar butyrate concentration in the cell cultures (B, D). Each curve is the linear regression (best-fit) curve. (Δ), Control; (▼), vegetable 3; (◇), tomato; (●), yeast; (□), linseed; (▲), vegetable 2; (■), inulin; (▽), soya; (○), wheat. The values are means of three to four independently reproduced experiments, with standard deviations represented by vertical bars. Significant differences are specified in Table 2.

the conclusion that fibre intake and colon cancer are inversely associated (Fuchs *et al.* 1999; Pietinen *et al.* 1999). Intervention trials have also been unconvincing, as has been reported for the recurrence of colonic adenomas which were not preventable by intervention with dietary fibres (McKeown-Eyssen *et al.* 1994; Alberts *et al.* 2000; Schatzkin *et al.* 2000). Conversely, recent large trials

support a protective role for high-fibre diets against cancer. One prospective observational study with 519 978 adults, chosen from a variety of sample populations at twenty-two centres across Europe (European Prospective Investigation into Cancer and Nutrition), shows that high fibre intake is associated with a 25% reduction in colon cancer risk (Bingham *et al.* 2003). Furthermore,

**Table 2.** Growth-inhibitory concentrations for all samples†  
(Mean values with their standard errors)

| Calculated as percentage sample | HT29 clone 19A cells         |       |   |                               |      |   | HT29 parent cells            |       |   |                               |      |   |
|---------------------------------|------------------------------|-------|---|-------------------------------|------|---|------------------------------|-------|---|-------------------------------|------|---|
|                                 | Supernatant EC <sub>50</sub> |       |   | SCFA-mixture EC <sub>50</sub> |      |   | Supernatant EC <sub>50</sub> |       |   | SCFA-mixture EC <sub>50</sub> |      |   |
|                                 | Mean                         | SE    | n | Mean                          | SE   | n | Mean                         | SE    | n | Mean                          | SE   | n |
| 1. Control                      | 28.31                        | 11.33 | 3 | 14.33                         | 1.16 | 3 | 53.65                        | 247.6 | 3 | 12.07                         | 0.25 | 3 |
| 2. Vegetable 3                  | 13.40                        | 1.63  | 3 | 12.37                         | 2.40 | 3 | 43.39                        | 42.79 | 3 | 16.81                         | 0.73 | 3 |
| 3. Tomato                       | 4.47*                        | 0.18  | 4 | 5.84                          | 0.14 | 4 | 4.67*                        | 0.26  | 4 | 13.30†                        | 0.32 | 4 |
| 4. Yeast                        | 5.14*                        | 0.34  | 3 | 8.41                          | 0.25 | 3 | 9.63†                        | 1.35  | 3 | 8.37                          | 0.17 | 3 |
| 5. Linseed                      | 2.96*                        | 0.23  | 4 | 5.86                          | 0.22 | 4 | 3.27*                        | 0.23  | 4 | 6.27                          | 0.26 | 4 |
| 6. Inulin                       | 4.15*                        | 0.16  | 3 | 6.78                          | 0.32 | 3 | 7.48†                        | 0.44  | 3 | 5.92                          | 0.28 | 3 |
| 7. Vegetable 2                  | 2.62*                        | 0.07  | 3 | 7.03                          | 0.28 | 3 | 5.37*†                       | 0.19  | 3 | 8.78†                         | 0.32 | 3 |
| 8. Soya                         | 3.80*                        | 0.19  | 4 | 5.54                          | 0.14 | 4 | 4.63*                        | 0.16  | 4 | 6.75†                         | 0.20 | 4 |
| 9. Wheat                        | 2.46*                        | 0.16  | 4 | 4.74                          | 0.23 | 4 | 2.83                         | 0.13  | 4 | 4.36                          | 0.19 | 4 |
| Mean 1 §                        | 3.66*                        | 0.38  | 7 | 6.31                          | 0.45 | 7 | 5.41                         | 0.91  | 7 | 7.68                          | 1.09 | 7 |
| Mean 2 §                        | 4.87*                        | 0.68  | 7 | 11.1                          | 1.01 | 7 | 6.54*                        | 0.95  | 7 | 12.55                         | 1.58 | 7 |

EC, effective concentration; SCFA, short-chain fatty acids.

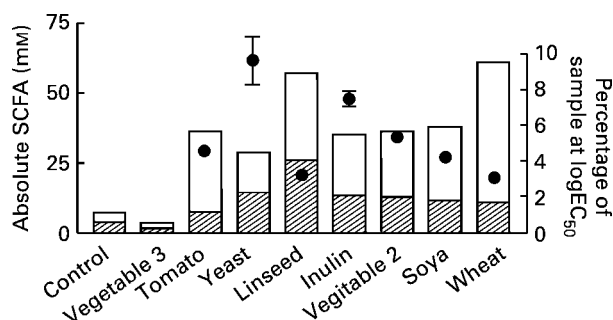
\* Mean value, within a cell type, was significantly different from that for the SCFA mixture ( $P < 0.05$ ) (two-way ANOVA and Bonferroni's *post hoc* test to compare replicate means by row).

† Mean Log EC<sub>50</sub> value, for supernatant or SCFA-mixture, was significantly different to that for the HT29 clone 19A cells ( $P < 0.05$ ) (two-way ANOVA and Bonferroni's *post hoc* test to compare replicate means by row).

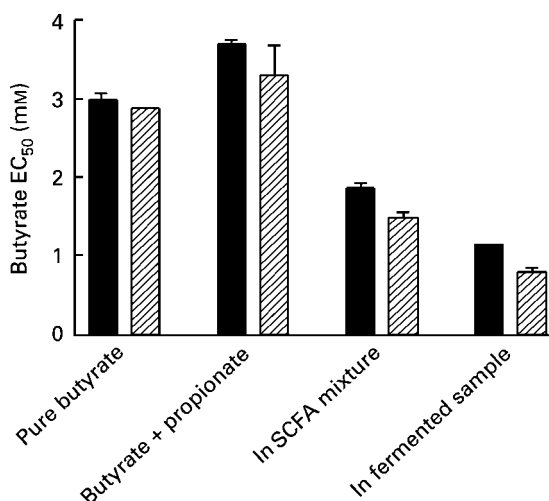
‡ Based on 'percentage sample' (% v/v) in incubation mixture.

§ Mean Log EC<sub>50</sub> of samples numbered 3 to 9; 'mean 1' values were calculated from best-fit curves, set from top (constant 100) to bottom (variable). In comparison, the 'mean 2' values are corresponding EC<sub>50</sub> values from best-fit curves set from top (=100) to bottom (=0).





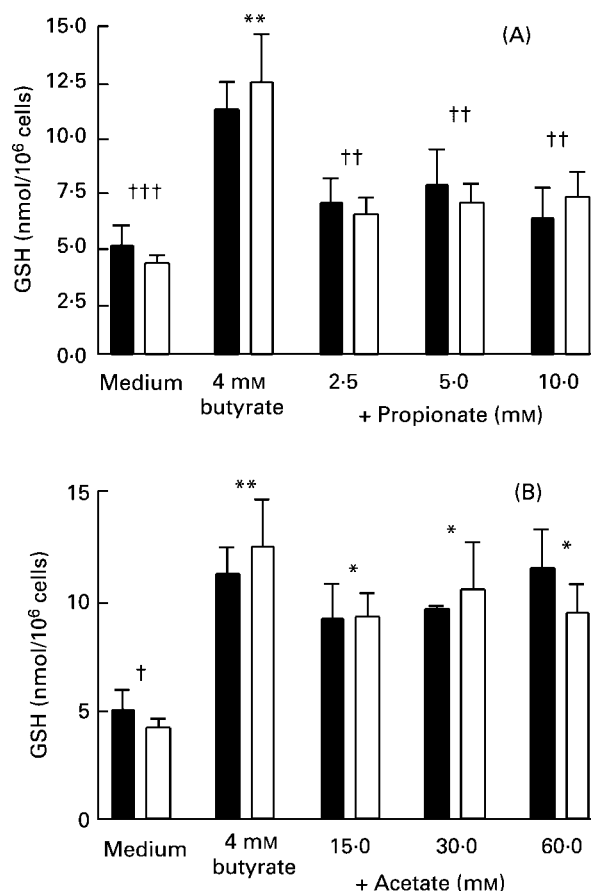
**Fig. 4.** Direct comparison of absolute butyrate and propionate concentrations to the amounts of samples needed to achieve inhibitory concentrations (% v/v) in HT29 cells. The volumes of control or vegetable 3 that would be necessary to achieve the inhibitory concentration exceed 10% (54 and 43%, respectively) and are therefore not plotted in this Fig. The respective data are presented in Table 2. The values are means, with standard errors represented by vertical bars. (□), Butyrate; (▨), propionate; (●), log effective concentration ( $EC_{50}$ ) (% sample).



**Fig. 5.** Inhibitory concentrations for butyrate calculated to be effective when acting on the cells alone, or in combination with propionate, propionate + acetate, or within a complete fermentation sample. The data of fermentation samples are taken from Fig. 3. The values are means, with standard deviations represented by vertical bars. (■), HT29 parent cells; (▨), HT29 clone 19A cells; EC, effective concentration.

a case-control evaluation study with 43 611 participants in a multi-centre randomised trial of different methods for screening for early cancer (Prostate, Lung, Colorectal, and Ovarian Cancer Screening project) shows that subjects with colonic adenomas have lower fibre intakes, particularly from grains, cereals and fruits (Peters *et al.* 2003).

These contradictory findings might be attributed to the undifferentiated way that dietary fibre is characterised and to the lack of consideration of the mechanisms involved. The original dietary fibre hypothesis was based on the finding of a low incidence of colorectal cancer in rural, black Africans, whose diet contained large amounts of unprocessed plants as food (Burkitt, 1969). The dietary fibre quality of these foods, however, is quite different from present-day processed fibre. They may have quite different bulking effects in the gut (Ferguson *et al.*



**Fig. 6.** Modulation of cellular glutathione (GSH) levels after incubation (72 h) with combinations of short-chain fatty acids. A constant concentration of butyrate (4 mM) which increases the levels of the control 2-fold was the basis, and propionate (A) or acetate (B) were added in amounts within the potential physiological concentration range. (■), HT29 clone 19A cells; (□), HT29 parent cells. The values are means, with standard errors represented by vertical bars. One-way ANOVA with the multiple-comparison *post hoc* test were used to calculate the differences from the medium control ( $*P < 0.05$ ,  $**P < 0.01$ ), and from the butyrate control ( $\dagger P < 0.05$ ,  $\dagger\dagger P < 0.01$ ,  $\dagger\dagger\dagger P < 0.001$ ); the latter was measured in concurrent experiments during the same experimental period.

2001), may lead to different SCFA patterns (Kobayashi & Fleming, 2001), or contain different amounts of nutrients and phytochemicals, that have been linked to cancer protection (World Cancer Research Fund and American Institute for Cancer Research, 1997). It is conceivable that all three of these traits could have quite different impacts on cancer risk reduction.

It is not possible to study bulking effects using the methods described in the present study. Also, *in vitro* fermentation conditions do not allow the complete representation of the physiological situation, including differences in residence time and multiple interaction effects, but the study has at least been able to obtain some new information on suppressing activities by fermentation products from a number of different dietary fibres sources. Thus, the comparison of the anti-proliferative effect in the two cell lines revealed that for vegetable 2 (fermentation sample and SCFA mixture), inulin and yeast (fermentation samples) as well as for soya and tomato (SCFA mixture)

**Table 3.** Glutathione S-transferase and alkaline phosphatase activities in HT29 cells as markers of chemoprevention and differentiation, respectively\* (Mean values with their standard errors)

| Tested samples                  | Amount (% v/v) | Viability (%) |      |   | Activity of glutathione S-transferase |      |   |                         |      |   | Activity of alkaline phosphatase |      |   |               |      |   |
|---------------------------------|----------------|---------------|------|---|---------------------------------------|------|---|-------------------------|------|---|----------------------------------|------|---|---------------|------|---|
|                                 |                | Mean          | SE   | n | nmol/min per 10 <sup>6</sup> cells    |      |   | nmol/min per µg protein |      |   | mU/10 <sup>6</sup> cells         |      |   | mU/mg protein |      |   |
|                                 |                |               |      |   | Mean                                  | SE   | n | Mean                    | SE   | n | Mean                             | SE   | n | Mean          | SE   | n |
| Fermented supernatant fractions |                |               |      |   |                                       |      |   |                         |      |   |                                  |      |   |               |      |   |
| Medium                          |                | 91            | 0.85 | 4 | 40.19                                 | 2.06 | 4 | 0.39                    | 0.05 | 4 | 0.28                             | 0.07 | 4 | 2.14          | 0.25 | 4 |
| Control or blank                | 5.00           | 60            | 17.8 | 3 | 65.11                                 | 28.3 | 3 | 0.45                    | 0.08 | 4 | 0.36                             | 0.03 | 3 | 2.38          | 0.47 | 4 |
| Inulin                          | 3.75           | 93            | 1.51 | 4 | 49.28                                 | 4.66 | 4 | 0.37                    | 0.03 | 4 | 0.35                             | 0.10 | 4 | 2.12          | 0.28 | 4 |
| Soya                            | 2.50           | 86            | 6.66 | 4 | 41.14                                 | 3.63 | 4 | 0.26                    | 0.06 | 4 | 0.29                             | 0.05 | 4 | 1.55          | 0.36 | 4 |
| Wheat                           | 1.50           | 94            | 1.01 | 4 | 44.14                                 | 4.91 | 4 | 0.29                    | 0.02 | 4 | 0.32                             | 0.06 | 4 | 1.91          | 0.28 | 4 |
| SCFA mixtures                   |                |               |      |   |                                       |      |   |                         |      |   |                                  |      |   |               |      |   |
| NaCl                            |                | 91            | 2.59 | 4 | 37.36                                 | 1.36 | 4 | 0.32                    | 0.04 | 4 | 0.27                             | 0.07 | 4 | 1.87          | 0.31 | 4 |
| 'Control' SCFA                  | 5.00           | 95            | 1.42 | 4 | 36.47                                 | 2.25 | 4 | 0.30                    | 0.04 | 4 | 0.24                             | 0.05 | 4 | 1.71          | 0.31 | 4 |
| 'Inulin' SCFA                   | 3.75           | 86            | 7.45 | 4 | 31.67                                 | 3.23 | 4 | 0.39                    | 0.08 | 4 | 0.26                             | 0.07 | 4 | 2.30          | 0.38 | 4 |
| 'Soya' SCFA                     | 2.50           | 96            | 2.52 | 4 | 43.14                                 | 4.36 | 4 | 0.30                    | 0.05 | 4 | 0.26                             | 0.07 | 4 | 1.50          | 0.19 | 4 |
| 'Wheat' SCFA                    | 1.50           | 95            | 1.59 | 4 | 37.50                                 | 1.42 | 4 | 0.76                    | 0.39 | 4 | 0.27                             | 0.06 | 4 | 3.77          | 1.22 | 4 |

SCFA, short-chain fatty acids; EC, effective concentration.

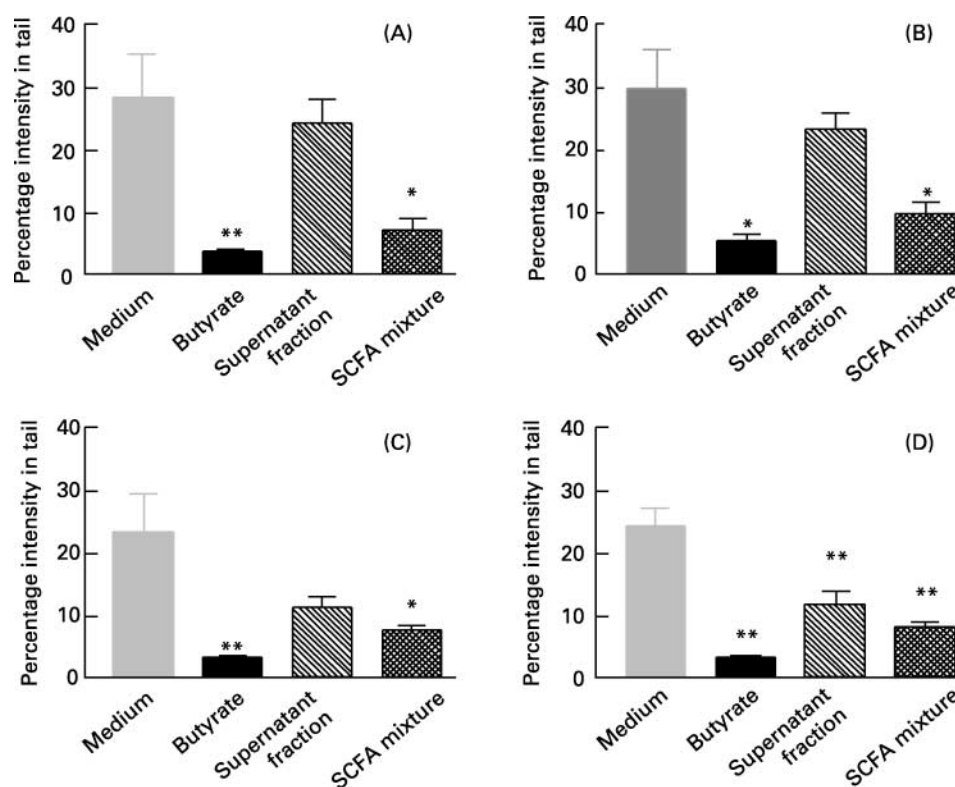
\* Treatment was with SCFA mixtures and fermentation samples from three selected samples and the fermentation 'control' (no fibre). Concentrations were approximately 75% of the EC<sub>50</sub> volumes, to avoid extensive toxicity and results are presented both on the basis of cells as well as on the basis of cellular protein content. There were no statistically significant differences.

the differentiated clone cells were more sensitive to the products than the HT29 parent cells, which are highly transformed. This could mean that the samples are beneficial at earlier stages of cancer development, a theory which needs additional verification using appropriate cell lines representing even earlier stages of colon carcinogenesis, such as adenoma cells (Schäferhenrich *et al.* 2003a).

Another group of significant findings was the magnitude of growth inhibition caused by butyrate in relation to SCFA mixtures; and the magnitude of effect caused by the complete fermentation sample in relation to the pure SCFA mixtures. In the latter case, the complete fermentation products were more capable of inhibiting the growth of HT29 clone cells than the synthetic SCFA mixtures. The so-called 'added value' of the complete fermentation samples probably reflects the growth-inhibitory properties of a high number of other plant ingredients and nutrients (World Cancer Research Fund and American Institute for Cancer Research, 1997). Given the assumption that *in vivo* these secondary compounds will also reach the colon lumen (directly or indirectly by systemic routes), the findings are in line with the recognition that additional components such as the antioxidant and anti-mutagenic compounds hydroxycinnamic acids or polyphenols contribute to anti-cancer mechanisms in the gut (Ferguson & Harris, 2003). Glycosylated polyphenols, for example, could be released into the colon during microbial fermentation, and undergo a further step of degradation, for example, by bacterial β-glycosidase yielding aglycones and glycosides. The liberation of aglycones will affect biological activity (Day *et al.* 2000). Previous studies have, for example, shown that both glycosides (anthocyanins) and aglycones (anthocyanidins) are effective antioxidants (Pool-Zobel *et al.* 1999), whereas only the aglycones inhibited HT29 cell growth *in vitro* (Briviba *et al.* 2001a).

Here it has also been observed, using the highly transformed cell line HT29, that the differences are especially apparent for a selected subset of samples, each of which theoretically could give rise to aglycones that have already been shown to have biological properties in these cells. These are isoflavones from soya (Pool-Zobel *et al.* 2000), lignans from linseed (Pool-Zobel *et al.* 2000), polyphenols from vegetable 2 (Duthie *et al.* 1997), and carotenoids from tomato (Vosseler, 2000; Briviba *et al.* 2001b). Interestingly, the fermentation sample from inulin and its corresponding SCFA mixture were of equal potency in mediating growth inhibition, as was yeast and the SCFA mixture. Neither of these two products contains specific antioxidant phytoprotectants, since they are made up mainly of oligofructose and glucans, respectively. The overall results therefore provide preliminary, but novel, information that complete fermentation samples from plant foods containing a variety of different phytoprotectants are more active than their corresponding pure SCFA mixtures. This could mean that fibre derived from vegetables has an 'added value' for inhibiting the growth of transformed colon tumour cells, whereas the activities of pure fibre sources (inulin and yeast) is mainly based on the SCFA produced during fermentation.

In this context, the question that needed to be answered was how much of the overall activity can be attributed to



**Fig. 7.** 4-Hydroxynonenal (HNE)-induced DNA damage ( $150\ \mu\text{M}$  for 30 min) after pre-incubation (72 h) of the cells with butyrate, reconstituted short-chain fatty acids (SCFA) and fermentation samples from control (A), fermented inulin (B), wheat (C) and soya (D). Values are means, with standard errors represented by vertical bars. Mean value was significantly different to the 'HNE-medium' control (pre-treatment with medium and second treatment with  $150\ \mu\text{M}$  HNE): \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ) (one-way ANOVA with Bonferroni's multiple comparison *post hoc* test).

butyrate, and in which manner can other SCFA modulate HT29 survival *in vitro*. To date, only relatively few data were available on how physiological concentrations of SCFA mixtures affect different parameters in human colon cells. Mariadason *et al.* (2001) investigated the abilities of a panel of SCFA to induce AP activity in undifferentiated Caco-2 cells, and measured the rate of consumption by which the cells took up the individual substances. Even though the SCFA were consumed by the cells at similar rates, they had different magnitudes of biological activities. Butyrate, and to a lesser extent propionate, induced AP activity whereas acetate had no effect (Mariadason *et al.* 2001). This is in line with previous results on the modulation of cell growth, where butyrate and propionate were most effective, and acetate was least active. The present studies, however, also investigated specific interactions among the three major SCFA. The SCFA mixtures prepared to mimic the SCFA concentrations of the fermentation samples had 10-fold different SCFA concentrations and 3- to 4-fold different potency to prevent cell growth (comparison of activities from all SCFA samples in HT29 cells). Based on butyrate concentrations (mM), however, the growth curves overlapped showing that the growth-inhibitory properties of SCFA are directly related to the butyrate content. Butyrate's impact on cell proliferation, however, is additive with propionate (but not with acetate), since propionate also inhibits cell growth at low concentrations. Thus, the

SCFA mixtures were much more effective inhibitors of cell growth than what was to be expected on account of the available butyrate. This means that both butyrate and propionate are responsible for this important parameter of growth inhibition of transformed cells.

One property of butyrate that could be considered as a 'double-edged sword' is that it enhances GST (Kirlin *et al.* 1999), a mechanism by which the cells develop chemoresistance against the genotoxic risk factor HNE (Ebert *et al.* 2001). If this property occurred *in vivo*, there would be a positive selection pressure for the target cells. The mechanism would probably be beneficial for the host, if induction occurred in non-transformed cells, as has been demonstrated indirectly, after feeding dietary fibre to rats (Treptow-van Lishaut *et al.* 1999). In contrast, the induction of GST activity and GSH levels in tumour cells is not a marker of chemoprevention. It may be reflecting an increase of the most abundant GST in colon cells, namely GSTP1 which is also regarded as a tumour marker when it is overexpressed in colon carcinoma (Miyanishi *et al.* 2001). However, recent studies have also shown that the increase of GST activity is due substantially to the induction of GSTA1/2 and GSTM2, which are down regulated in tumour cells (Peters *et al.* 1992) and thus are promising indicators of chemoprevention. These reflections are hypothetical since neither SCFA mixtures nor the fermentation samples modulated GST-GSH or AP activities in HT29 cells. The samples were investigated at

concentrations, which provide less than 2 mM-butyrate to the cells. The concentrations are too low to significantly induce GST activity for which 4 mM-butyrate is needed under the same treatment and culture conditions. However, the SCFA mixtures were effective in protecting against HNE genotoxicity, although less so than butyrate. Possibly the conditions sufficed to induce GSTA4-4, the most important isoenzyme in HNE deactivation (Board, 1998; Bruns *et al.* 1999), or other GST which could inactivate HNE due to overlapping substrate specificities (Berhane *et al.* 1994). While these possibilities are under current investigation, it still remains unclear why the fermentation samples, which contain identical levels of SCFA, are not as effective in mediating chemoresistance, especially for the example of inulin and its corresponding SCFA mixture. Apparently other as yet unknown factors produced during bacterial metabolism are counteracting the modulation of HNE activity.

In summary, the present study is one of the first of its kind to compare the biological activities of complex mixtures produced from fermentation of different sources of dietary fibres. It has the limitations of any *in vitro* study and assumes the unlikely condition that all components reach the colon in sufficient amounts to be fermented in a way that equitoxic concentrations are achieved. However, the study demonstrates clearly that physiological and toxicological responses in colon tumour cells are mediated by the SCFA, of which butyrate has a predominant role. However, butyrate does not act alone and propionate, in particular, contributes an approximately equal amount to the growth-inhibitory effects of SCFA mixtures. The study also demonstrates that in addition to SCFA, other ingredients of soya, vegetable fibre, linseed and tomato add to the growth-inhibitory properties of butyrate and propionate. In contrast, the induction of GST activity and the mediation of chemoresistance toward HNE are either completely or partially prevented by the additional SCFA and by as yet unidentified products of microbial fermentation. Translated to the *in vivo* situation, the results suggest that fermentation products can suppress the growth of tumour cells by a number of components including butyrate, propionate, and unidentified phytochemicals.

Finally the present study shows that our strategy can be used to study dietary fibres for biological activities using cell culture systems. Further characterisation of the responses, validation of additional endpoints, and replacing the tumour cells by primary human colon cells (Schäferhenrich *et al.* 2003b) or cells of a human colon adenoma cell line (Schäferhenrich *et al.* 2003a) will yield a very useful tool to pre-screen new food products for functional properties and to better understand how dietary fibres can affect colon cells in different stages of development, differentiation or transformation.

### Acknowledgements

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