British Journal of Nutrition (2011), **105**, 678–687
doi:10.1017/S0007114510004320

Modification of an *in vitro* model simulating the whole digestive process to investigate cellular endpoints of chemoprevention

Katrin Stein[1]*, Anke Borowicki[1], Daniel Scharlau[1], Kerstin Scheu[2], Gerald Brenner-Weiß[2], Ursula Obst[2], Jürgen Hollmann[3], Meinolf Lindhauer[3], Norbert Wachter[4] and Michael Glei[1]

[1]*Department of Nutritional Toxicology, Institute for Nutrition, Friedrich-Schiller-University Jena, Dornburger Strasse 24, 07743 Jena, Germany*
[2]*Department of Microbiology of Natural and Technical Interfaces, Research Center Karlsruhe, Institute of Functional Interfaces (IFG), Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany*
[3]*Max Rubner-Institute, Federal Research Institute of Nutrition and Food, Schützenberg 12, 32756 Detmold, Germany*
[4]*Kampffmeyer Food Innovation GmbH, Trettaustr. 32-34, 21107 Hamburg, Germany*

(Received 19 May 2010 – Revised 23 September 2010 – Accepted 24 September 2010 – First published online 28 January 2011)

Abstract

In vitro gut fermentation systems are relevant tools to study health benefits of foodstuffs. Most of them are commonly used to investigate the degradation of nutrients or the development of gut flora. Using these models, strong cytotoxic effects of the resulting samples on cultured cells were observed. Hence, the aim of the present study was to develop a modified *in vitro* fermentation model that simulates the whole digestive tract and generates fermented samples that are suitable for testing in cell culture experiments. Wholemeal wheat flour (wwf) was digested and fermented *in vitro* with a fermentation model using different ox gall concentrations (41·6 and 0·6 g/l). The resulting fermentation supernatants (fs) were characterised for metabolites and biological effects in HT29 cells. The fermentation of wwf increased chemopreventive SCFA and decreased carcinogenic deoxycholic acid (DCA). The strong cytotoxic effects of the fs, which were partly due to cholic acid and DCA, were diminished by lowering the ox gall concentration, allowing the use of the samples in cell culture experiments. In conclusion, an *in vitro* digestion model, which can be used to study the effects of foodstuffs on chemoprevention and gut health in colon cells, is introduced and its physiological relevance is demonstrated.

Key words: Chemoprevention: Deoxycholic acid: Fermentation: SCFA: Wheat

Several epidemiological studies have suggested that diet and lifestyle play a major role in carcinogenesis, especially in colon cancer[1,2]. The gastrointestinal tract (GIT) is continuously exposed to xenobiotics and endogenous metabolites, which can cause colorectal cancer or other diseases of the colon such as inflammatory bowel disease. Thus, chemoprevention of colorectal cancer is a major concern for improving public health. The consumption of dietary fibre from fruits and grains might be associated with a reduced risk of colorectal cancer development, whereby high but also lower amounts may exert beneficial effects[3,4]. Nevertheless, it is apparent that more detailed studies are needed on how different types of foods and dietary fibres contribute to gut health and how they may act on a molecular basis. In particular, it is of interest to better understand overall metabolism, the roles of individual compounds formed during the process of gut fermentation and how these fermentation metabolites interact with each other in a chemopreventive mode of action with colon epithelial cells. Therefore, to characterise the *in vivo* situations more precisely, it makes sense to determine the underlying mechanism *in vitro*. *In vitro* gut fermentation systems are thought to reflect conditions in the GIT which make them a relevant tool, with the advantages of low costs and relatively easy performance, to study health benefits and risks of foodstuffs and nutrients, metabolites of food digestion as well as effects on the colonic microflora. To determine how the intestinal epithelium is influenced by different gut metabolites in detail, it is useful to investigate the effects of fermentation samples in colon cell culture experiments. Only a few such cell culture studies are available, and most of them investigated the effects of fermentation samples from single substances, in particular dietary fibre, obtained from basic *in vitro* fermentation models that only mimic the large intestine[5–8]. Those studies highlighted the positive

Abbreviations: CA, cholic acid; DCA, deoxycholic CA; fs, fermentation supernatants; GIT, gastrointestinal tract; wwf, wholemeal wheat flour.

* **Corresponding author:** K. Stein, fax +49 3641949672, email stein.katrin@googlemail.com

effects of fermentation samples on colon cancer prevention by mechanisms that could retard tumour promotion and progression. The research of complex foodstuffs (e.g. wheat flour) on colon cancer prevention is more difficult because next to the fermentation process, the digestion and absorption of nutrients should be additionally taken into account. So far, detailed evidence is missing how the resulting fermentation products of complex foods interact with human colon cells.

Several *in vitro* models including a simple single-batch fermentation system described by Barry *et al.*[9] as well as more complex multistage continuous models[10–14], which can simulate selected or all passages of the whole digestive tract, have been developed. Since samples from previous *in vitro* multistage continuous systems have been shown to have cytotoxic effects in cell culture experiments[7], they are only used to elucidate the role of microbiota in the metabolism of all non-digestible parts of the diet[15] and to analyse fermentation products, e.g. SCFA, bile acids and NH_3[13]. Fassler *et al.*[7] suggested that the high cytotoxic activity of the fermentation samples is probably due to compounds (especially bile acids) added during *in vitro* fermentation which made them unsuited for any of the cell-based bioassays for cancer. In addition, they also showed a high cytotoxicity of the bile acid deoxycholic acid (DCA)[7]. Moreover, several other researchers have reported that bile acid concentration is highly correlated with the cytotoxicity of faecal water[16–18]. So far, information about a toxic influence of other compounds required during the process does not exist.

Therefore, the aim of the present study was to establish a new *in vitro* model, simulating the whole GIT based on two previously designed models by Barry *et al.*[9] and Aura *et al.*[19], suitable for subsequent cell culture experiments. A reduction of the added ox gall concentration to 0·6 g/l in our newly established model simulating the whole digestive tract is thus a critical parameter to use these samples in cell culture experiments. Therefore, the samples obtained using an optimised *in vitro* model protocol may be used to study the effects of different foodstuffs on colon cell lines and can therefore improve the outcome of *in vitro* studies. Moreover, fermentation products (SCFA and bile acids), generated by using different *in vitro* model conditions, were determined, and the influence of the samples on the number of HT29 colon cancer cells was measured. Furthermore, the efficiency of digestive enzymes and dialysis, which simulates the absorption of small molecular metabolites, was investigated.

Materials and methods

Dietary fibre sources

Wholemeal wheat flour (wwf) from Kampffmeyer Food Innovation GmbH (Hamburg, Germany) was used as a source of dietary fibre. The composition of wwf was reported by Borowicki *et al.*[20]. Synergy1®, a commercially available mixture of inulin enriched with oligofructose, was obtained from Orafti (Tienen, Belgium). To maintain the stability of samples, aliquots were prepared and stored in air- and light-proof flasks at 4°C.

Fermentation of dietary fibre sources

Wwf was digested and fermented *in vitro* in a batch culture system according to Aura *et al.*[19,20] and Barry *et al.*[9] with some modifications (Fig. 1). In detail, simulation of the

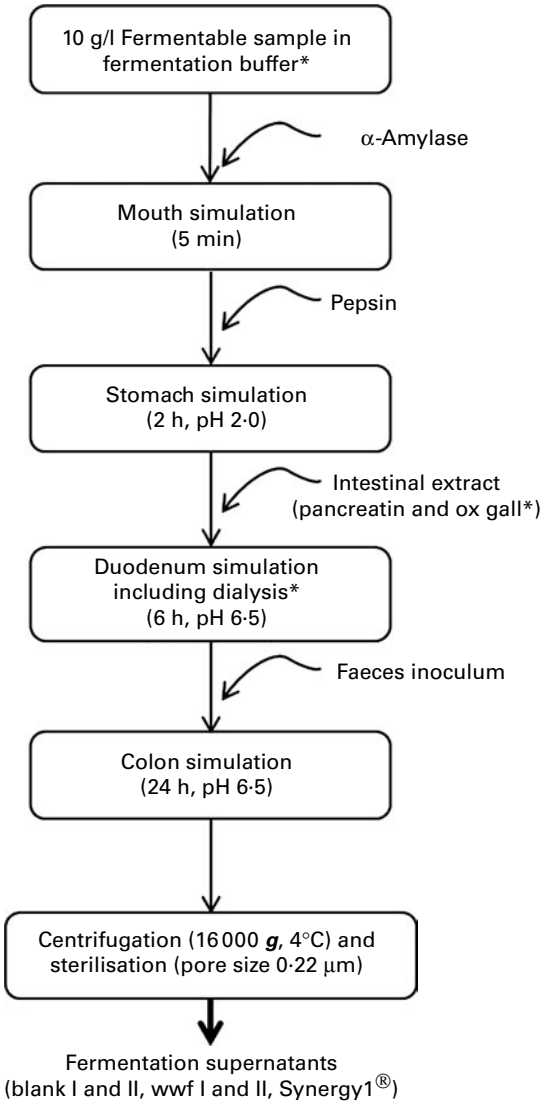

Fig. 1. Procedure of the *in vitro* fermentation method according to Aura *et al.*[19,20] and Barry *et al.*[9] with some modifications (denoted by *). In detail, simulation of the upper gastrointestinal tract designed by Aura *et al.*[19] was modified by the insertion of a dialysis step for the removal of the digested products and by the change of the amount as well as the solvent for the samples before mouth simulation. So, the samples were dissolved according to the protocol designed by Barry *et al.*[9] using a potassium phosphate fermentation buffer (0·1 M, pH 7·0) instead of a nutritive medium. The faecal control (blank) and wholemeal wheat flour (wwf) were digested and fermented using 41·6 g/l (I) or 0·6 g/l (II) of ox gall in the intestinal extract. Synergy1® was digested and fermented using 0·6 g/l (II) of ox gall in the intestinal extract.

upper GIT designed by Aura *et al.*[19] was modified by the insertion of a dialysis step for the removal of the digested products and by the change of the amount as well as the solvent for the samples before mouth simulation. So, the samples were dissolved according to the protocol designed by Barry *et al.*[9] using a potassium phosphate fermentation buffer (0·1 M, pH 7·0) instead of a nutritive medium. Two fermentation series, with different ox gall concentrations (fermentation I, 41·6 g/l; fermentation II, 0·6 g/l) in the intestinal extract for simulation of the small intestine, were conducted. Dietary fat accounts for 30 % of the body's energy requirement per day considering a balanced diet. This results in a daily secretion of 20–30 g bile acids during the digestion of foods containing a moderate fat content[21]. Hence, with regard to fat content and amount of the applied wheat source, a theoretical value of 0·6 g/l ox gall in the intestinal extract is required to achieve more physiological bile acid concentrations during the *in vitro* simulation of digestion. Moreover, for the statistical evaluation of the fermentation procedure, the well-characterised dietary fibre source Synergy1® was digested and fermented *in vitro* in three repetitions.

Wwf was weighed (0·5 g fermentable material) into glass bottles and mixed with 10 ml potassium phosphate buffer (0·1 M, pH 7·0). A sample without wwf was used as a control (blank). All the following incubations were performed at 37°C in a continuously shaking water-bath. Salivary α-amylase (Sigma, Taufkirchen, Germany) diluted to 1000 U/ml with 20 mM-sodium phosphate buffer (17·36 U/sample) and 0·85 % (w/v) NaCl were added, and the samples were incubated for another 5 min. HCl solution (150 mM, 2·81 ml) was added to lower the pH to 2·0. Pepsin (Sigma) dissolved in 0·94 ml of 20 mM-HCl was added to each sample and incubated for 2 h at 37°C. An intestinal extract was prepared by dissolving 208 mg (fermentation I) or 3 mg (fermentation II) ox gall (Fluka-Sigma), respectively, and 2·6 mg pancreatin (Sigma) in 5 ml sodium bicarbonate buffer (11 mM, pH 6·5). This solution (5 ml) was added to each sample or blank, the pH was adjusted to 6·5 using NaOH solution (10 M) and the suspension (25 ml) was transferred into a dialysis tube (molecular weight cut-off 1000 Da) (Roth, Karlsruhe, Germany). The tubes were placed into glass bottles filled with 2 litres of the dialysis buffer (13·61 g potassium phosphate and 1·88 g sodium bicarbonate dissolved in distilled water, pH 6·5) and incubated for 6 h at 37°C under semi-anaerobic conditions. Semi-anaerobic conditions in the glass bottles were achieved by removing a part of the air with an injected cannula (0·5 bar for 1 min) and, subsequently, filling the bottles with the fermentation gas mixture (86 % N_2, 10 % CO_2 and 4 % H_2 at 37°C) via the cannula (0·8 bar for 1 min). After 15 min (seven cycles repeated), the cannulae were removed. At the end of the incubation, the suspension was transferred from the dialysis tube into a 500 ml glass bottle, and the pH of each sample was measured.

Thereafter, the *in vitro* fermentation was performed according to the method described by Glei *et al.*[5]. Faecal homogenate (25 ml) was mixed with the digested wheat samples (as previously described; final concentration of wheat sample: 10 g/l), and the pH was adjusted to 6·5 using NaOH solution (10 M). *In vitro* fermentation was conducted under anaerobic conditions which were achieved by removing the available air with an injected cannula (0·5 bar for 1 min). Subsequently, the bottles were filled with the fermentation gas mixture via the cannula (0·8 bar for 1 min). After 30 min (fifteen cycles repeated), the cannulae were removed and the fermentation suspensions were incubated for 24 h in a shaking water-bath at 37°C. Afterwards, the fermentation process was stopped by placing the suspensions on ice, and the pH was measured with a pH meter (Hydrus 300; Fisherbrand, Schwerte, Germany). Each sample was centrifuged (4200 ***g*** at 4°C) for 30 min. Supernatants were centrifuged again (4200 ***g*** at 4°C) for 15 min and stored at −80°C. Before sterilisation of the fermentation supernatants (fs), the samples were thawed quickly, aliquoted in 2 ml tubes and centrifuged (16 000 ***g*** at 4°C). Afterwards, the fs were sterilised by filtration (pore size, 0·22 μm) to obtain the final fs for use in cell culture experiments.

To investigate the functionality of digestion enzymes and dialysis during wwf digestion, the remaining suspensions, dialysates (dialysis buffer containing low-molecular (< 1000 Da) digested products and bile acids) and retentates (suspension in the dialysis tube), obtained by simulation of mouth to the small intestine using 0·6 g/l ox gall, were stored at −80°C until further analysis.

Determination of bile acids

Bile acids were determined in the intestinal extract, retentate as well as dialysate, and in all fs by HPLC-MS/MS. Therefore, the fs were diluted 1:10 using potassium phosphate buffer. In total, 20 μl cholic acid (CA)-d_4 (50 μg/ml) were added as an internal standard. The sample enrichment was performed by neutral solid-phase extraction with 200 mg 101 sorbent columns (Separtis, Grenzach-Wyhlen, Germany). The columns were preconditioned with 10 ml methanol, 15 ml distilled water and 5 ml potassium phosphate buffer. The sample was applied onto the cartridge and allowed to pass through by gravity and water jet vacuum pump. Afterwards, the cartridge was washed with 10 ml distilled water and dried with N_2, and the bile acids were eluted with 5 ml methanol. The eluted substances were dried at 45°C under N_2, and the residue was dissolved in 1 ml 10 mM-NH_4OAc–acetonitrile (50:50, v/v). HPLC-MS/MS was performed according to Burkard *et al.*[22] with an Agilent 1100 Series HPLC system (Agilent, Waldbronn, Germany). Bile acids were separated on an RP-C8 column (100 × 4 mm, 5 μm; MZ-Analysentechnik, Mainz, Germany). The injection volume was 20 μl. The mobile phase consisted of 10 mM-ammonium acetate

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buffer, pH 5·0, containing 0·012 % formic acid (eluent A) and acetonitrile (eluent B). The eluents were linearly changed from 70 % A and 30 % B to 30 % A and 70 % B within 38 min, held for 10 min, and finally adjusted to the original ratio of 70 % A and 30 % B within 2 min and held for 15 min to equilibrate the column. The column flow rate was set to 0·5 ml/min during the entire analysis. The detection of bile acids was performed with a tandem mass spectrometer API 4000 (Applied Biosystems/MDS SCIEX, Toronto, Canada) equipped with an ESI ionisation source operating in the negative mode at −4·5 kV at 450°C. Bile acids were recorded in the multiple-reaction-monitoring mode at m/z 407·2 → 343·2 CA, m/z 411·2 → 347·2 (CA-d_4) and m/z 391·2 → 345 DCA. A validation of the method was done using enriched standards from 0·02 to 2·0 mg/l (n 3). Repeatability, standard deviation, limit of detection and limit of quantification were calculated according to the German Standard DIN 32 645.

Analysis of glucose concentrations

The amount of glucose was analysed in the retentate and dialysate as well as in the starting raw material by high-performance anion-exchange chromatography on a DIONEX BioLC system (DIONEX Corporation, Sunnyvale, CA, USA) according to Hollmann & Lindhauer[23].

Determination of SCFA

SCFA in the fs were determined by GC combined with MS (GC/MS) as described by Wang *et al.*[24] with some modifications. Briefly, the fs were diluted 1:50 using potassium phosphate buffer to a final volume of 1 ml, and 2-ethyl butyric acid was added as an internal standard. The samples were acidified with 20 µl HCl solution (32 %), and SCFA were extracted by shaking the samples two times with 700 µl *tert*-butyl methyl ether. The organic phases were collected, and 1 ml of the respective organic phases was derivatised with 20 µl trimethylsulphonium hydroxide solution (Fluka, Buchs, Switzerland) in methanol (10 %, v/v) for 20 min at room temperature. In total, 2 µl aliquots of the derivatisated samples were injected into a GC-MS (Varian Saturn 2000) onto a Stabilwax-DA column (30 m × 0·25 mm internal diameter × 0·25 µm; Restek, Bellefonte, PA, USA). He gas was used as a carrier. The chromatographic conditions were as follows: 60°C held for 2 min, 10°C/min until 150°C, held for 1 min and 50°C/min until 240°C, held for 10 min. For the detection of the SCFA, the mass spectrometer was used in the full-scan mode. A method validation was carried out using enriched six-point calibration functions from 5 to 100 mg/l (n 3). Repeatability, standard deviation, limit of detection and limit of quantification were calculated according to the German Standard DIN 32 645.

Preparation of synthetic bile acid mixtures

The preparation of the synthetic mixtures of the two main bile acids, CA and DCA, contained in the fs blank and wwf of fermentation I and II was based on the analysis of bile acids in the fs I and II from our present experiments. Investigations using the synthetic mixtures were expected to reveal which cellular effects were caused by the bile acids in the fs. Therefore, sodium cholate hydrate (Sigma) and sodium deoxycholate (Sigma) in the determined concentrations were dissolved in Dulbecco's modified Eagle's medium (Gibco BRL, Eggenstein, Germany) cell culture medium supplemented with 10 % fetal calf serum.

Cell culture

The human colon adenocarcinoma cell line HT29 was established in 1964 by Fogh (Memorial Sloan-Kettering Cancer Centre, New York, NY, USA)[25] and was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells (passages 30–40) were maintained as a subconfluent monolayer cultured in a supplemented Dulbecco's modified Eagle's medium at 37°C in a 95 % humidified incubator (5 % CO_2). The cultured cells were trypsinised with 5 ml of trypsin–versene (1:10, v/v) for 4 min and subcultivated at dilutions of 1:5 to 1:10 in T_{75} flasks. In regular intervals, a mycoplasma test (MycoAlert™ Detection Kit; Lonza Rockland, Inc., Rockland, ME, USA) was performed, and contamination with mycoplasma was excluded.

Measurement of the cell number

The determination of cytotoxicity of different test substances mainly proceeds by indirect (e.g. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4′,6-diamidino-2-phenylindole (DAPI) assay) or direct measurements (e.g. trypan blue exclusion test) of the cell number as an accepted marker which is often used in *in vitro* studies[26–29]. We decided on the DAPI assay by which the amount of the measured DAPI-labelled DNA is related to the number of cells.

The number of colon cells was determined in ninety-six-well microtitre plates. At 24 h after seeding, the cells were incubated with 2·5–20·0 % (v/v) of all fs diluted in Dulbecco's modified Eagle's medium to determine cytotoxicity. After 24, 48 and 72 h, the cell number was measured as described[30]. Results were calculated on the basis of the medium control which was set to equal 100 %.

Statistical analysis

All experiments apart from the fermentation (I and II) of wwf were conducted independently at least three times. Means and standard deviations were calculated from at

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least three independent experiments. Differences were calculated with GraphPad Prism version 5.02 for Windows (GraphPad Software, Inc., San Diego, CA, USA) using one- or two-way ANOVA with Bonferroni's post-test with selected pairs or Student's *t* test. The statistical analyses used depended on the respective experimental design and are specified in the legends to the figures and tables.

Results

Bile acids in fermentation supernatants

It is important to note that the two applied ox gall concentrations contained different amounts of bile acids (fermentation I: 645 μM-CA, 423 μM-DCA; fermentation II: 9 μM-CA, 6 μM-DCA). Levels of bile acids, both CA and DCA, in the fs were in general higher than those in the applied intestinal extracts, due to the added faecal sample. High concentrations of CA were detected in fs blank I (1562 μM) and in fs wwf I (656 μM). The concentration of DCA was highest in the fs blank I (502 μM). The use of the low ox gall concentration resulted in a reduced amount of DCA in fs wwf II (2 μM) and in fs blank II (26 μM). Noteworthy is the fact that the fermentation of wwf reduced the potential toxic secondary bile acid DCA independently of the ox gall concentration (wwf I, 2 μM; wwf II, 2 μM).

SCFA and pH in fermentation supernatants

The fs were further characterised for their contents of SCFA and pH after 24 h anaerobic fermentation. The amount of SCFA in both fs wwf I and II was markedly increased in comparison with the respective fs blank (Table 1). The amount of butyrate was highest in fs wwf II. Furthermore, the relative proportion of butyrate slightly increased. Interestingly, the relative and absolute propionate concentrations in the samples of fs I were higher than those in the fs II samples, whereas butyrate levels increased in the latter. Fermentation of wwf resulted in a clearly decreased pH compared with the fs blank. Lowered pH may reflect the increased total amount of the SCFA by active microflora.

Table 1. Concentration and molar ratios of SCFA as well as pH in fermentation supernatants (fs) of the faecal control (blank) and wholemeal wheat flour (wwf) after fermentation I (41·6 g/l ox gall) and II (0·6 g/l ox gall)

	fs blank I	fs wwf I	fs blank II	fs wwf II
pH	6·61	5·19	6·54	5·09
SCFA (mM)				
Acetate	23·49	60·68	24·15	74·00
Propionate	17·61	53·38	12·15	23·67
Butyrate	5·62	17·85	9·48	30·30
Total SCFA (mM)	46·72	131·91	45·77	128·50
Ratio of SCFA (%)	50:38:12	46:40:14	53:27:20	58:19:23

Effects of fermentation supernatants on the cell growth of HT29

Fig. 2 shows the effects of the fs blank and fs wwf of fermentation I and II on the cell growth of HT29 cells. The treatment of HT29 cells with the fs of fermentation I (fs blank and fs wwf) and II (fs blank and fs wwf) resulted in a significant time- and dose-dependent decrease of surviving cells. In comparison, the effects of fs blank I were significantly different from those of fs blank II, whereby fs blank I was the most cytotoxic of all samples with an inhibitory concentrations leading to 50 % reduction of cell number of 14·1 (SD 1·2) % (Table 2) after 24 h of treatment. The reduced cytotoxicity of fs blank II with an inhibitory concentrations leading to 50 % reduction of cell number of 13·4 (SD 1·1) %, which was reached only after 48 h, indicates that residual ox gall must be involved in the reduction of the cell number. Moreover, fs blank I was more cytotoxic than the corresponding fs wwf I, whereas fs wwf II was more effective than fs blank II in the inhibition of cell growth.

Effects of synthetic mixtures of cholic acid and deoxycholic acid on the number of HT29 cells

To characterise the role of bile acids in the inhibition of cell growth, HT29 cells were treated with synthetic mixtures of CA and DCA, which were prepared according to their corresponding complex fs blank and fs wwf of fermentation I and II, respectively (Fig. 3). The number of HT29 cells was only decreased time dependently by the synthetic bile acid mixture of fs blank I (48 and 72 h). However, in comparison with the complex fs blank I (Fig. 2), it was significantly less effective. All other synthetic mixtures of complex fs (fs wwf I, fs blank II and fs wwf II) had no effect on the cell number.

Efficacy of digestion enzymes and dialysis

To investigate the efficiency of digestion and dialysis, we characterised the retentate and dialysate (0·6 g/l ox gall in the intestinal extract) for their contents of glucose and bile acids. Digestion of wwf resulted in a total increased amount of glucose in the dialysate (3·64 mg absolute) and retentate (12·64 mg absolute) compared with the raw material (0 mg). This demonstrates an effective enzymatic degradation of starch and at least partial dialysis of glucose during simulation of the small intestine. Furthermore, most of the amounts of the inserted bile acids, CA (blank, 510 μM; wwf, 380 μM) and DCA (blank, 330 μM; wwf, 260 μM) were found in the dialysates (blank: 360 μg CA, 280 μg DCA; wwf: 230 μg CA, 190 μg DCA), which also proofed the functionality of dialysis during simulation of the upper digestive tract.

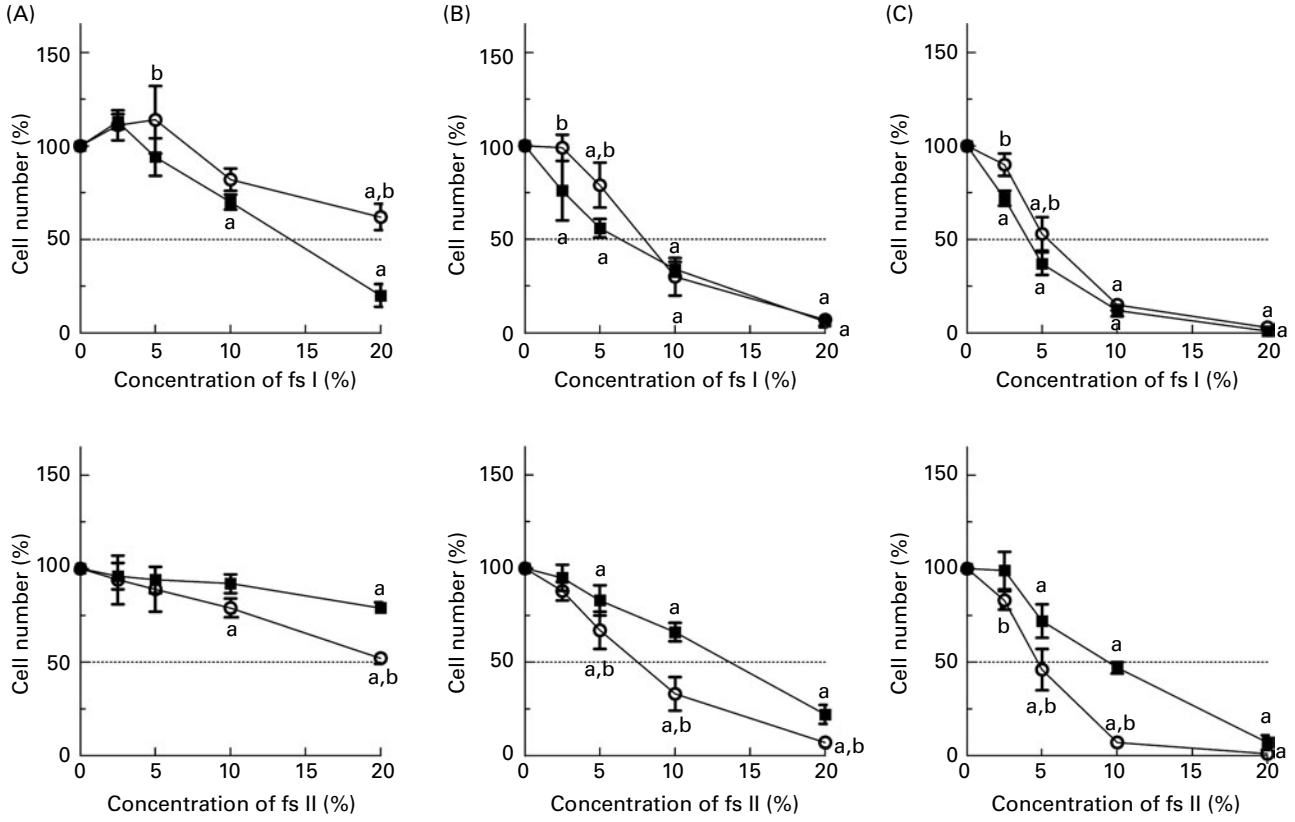

Fig. 2. Effects of fermentation supernatants (fs) I (upper graphs) and II (lower graphs) of the faecal control (blank, ■) and wholemeal wheat flour (wwf, ○) using 41·6 or 0·6 g/l of ox gall in the intestinal extract on the HT29 cell number after 24 h (A), 48 h (B) and 72 h (C) incubation. The signal of the medium control was set to equal 100 %. Values are means with standard deviations depicted by vertical bars (n 3). [a] One-way ANOVA with Bonferroni's post test with selected pairs was used to calculate the differences to the medium control ($P<0·05$). [b] Two-way ANOVA with Bonferroni's post test was used to calculate the differences between blank and wwf ($P<0·05$).

Statistical evaluation of fermentation II

In order to statistically validate the newly established fermentation protocol II, Synergy1®, a well-analysed dietary fibre, was fermented in three independent experiments according to the protocol described in Fig. 1. Key parameters, namely concentrations of SCFA and bile acids, were then analysed in the obtained fs. The fermentation of Synergy1® resulted in a strong and significant decrease of DCA (2·23 (SD 2·07) μM) and a non-significant decrease of CA (0·27 (SD 0·19) μM) compared with the fs blank (DCA, 36·06 (SD 10·27) μM; CA, 0·36 (SD 0·12) μM) (Table 3). The total SCFA concentration in the fs Synergy1® was about a threefold increase ($P<0·1$) in comparison with the blank. In addition, a significant increase of butyrate by approximately fourfold was observed (blank, 6·65 (SD 2·52) μM; Synergy1®, 27·28 (SD 2·64) μM). The values of the increase in total SCFA as well as butyrate were almost comparable to those obtained by the fermentation II from wwf (total SCFA, threefold; butyrate, threefold; Table 1), which also attributes to the use of the same amount of the fermentable substance of each sample. Thus, these results statistically support the reproducibility of the established protocol of fermentation II.

Discussion

SCFA production

Major metabolites of the gut flora-mediated fermentation of foodstuffs are SCFA, which result in a lower pH in the colon. Special attention is being paid to the ratio of SCFA, since they exhibit different physiological functions and effects. Various population survey data showed that

Table 2. Inhibitory concentrations leading to a 50 % reduction of cell number (EC_{50}) after incubation of HT29 cells with fermentation supernatants (fs) of the faecal control (blank) and wholemeal wheat flour (wwf) after fermentation I (41·6 g/l ox gall) and II (0·6 g/l ox gall)*

(Mean values and standard deviations, n 3)

	EC_{50} (%)							
	fs blank I		fs wwf I		fs blank II		fs wwf II	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
24 h	14·1	1·2	×		×		×	
48 h	6·3	1·1	8·6[b]	0·8	13·4[c]	1·1	7·7[b]	1·7
72 h	3·9	0·5	6·5[b]	0·5	9·8[c]	0·8	4·6[b]	0·4

×, EC_{50} was not achieved.

* Student's t test was used to calculate the differences between the respective fs blank (b: $P<0·05$) and between the respective sample of the fermentation I and II (c: $P<0·05$).

https://doi.org/10.1017/S0007114510004320 Published online by Cambridge University Press

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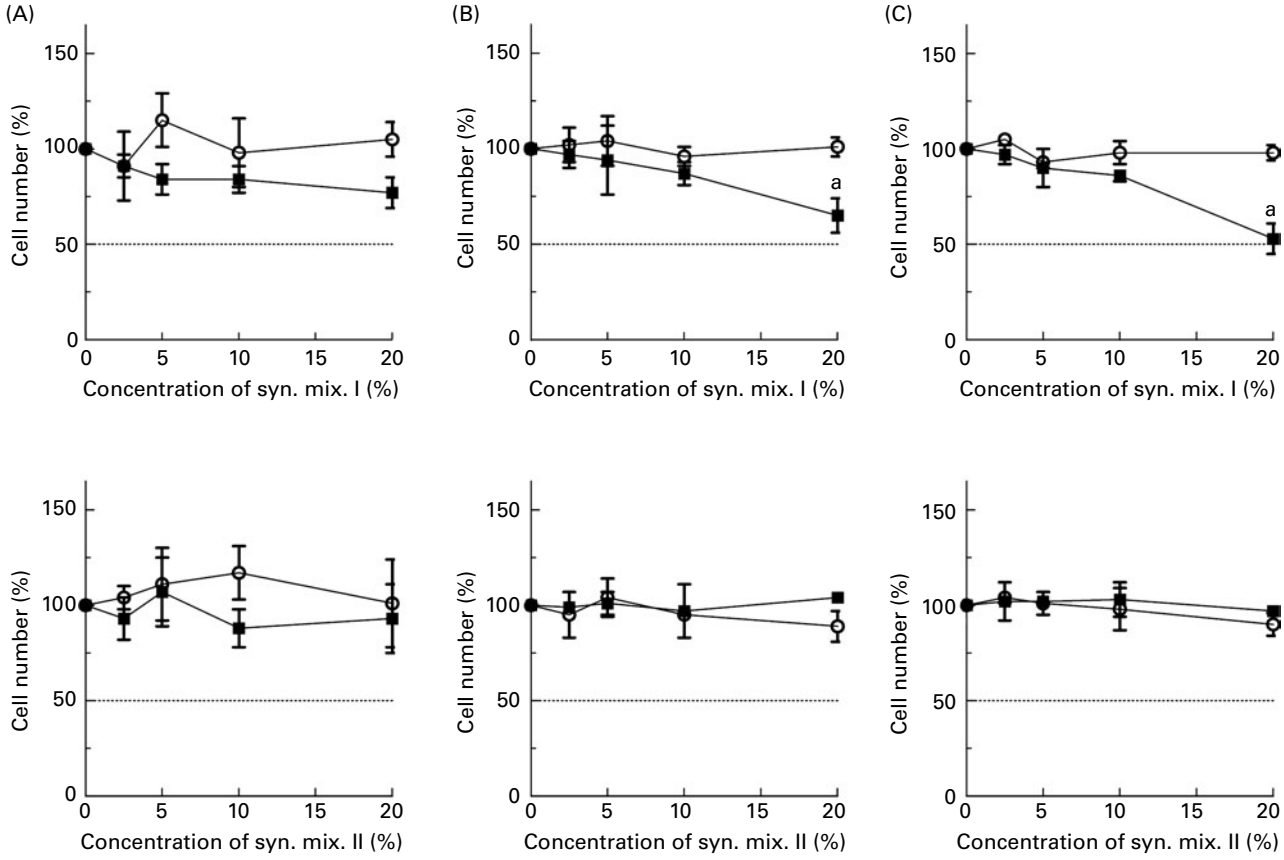

Fig. 3. Effects of corresponding synthetic mixtures (syn. mix.) of cholic acid and deoxycholic acid of the complex fermentation supernatants I (upper graphs) and II (lower graphs) of the faecal control (blank, ■) and wholemeal wheat flour (wwf, ○) using 41·6 or 0·6 g/l of ox gall in the intestinal extract on the HT29 cell number after 24 h (A), 48 h (B) and 72 h (C) incubation. The signal of the medium control was set to equal 100 %. Values are means and standard deviations depicted by vertical bars (n 3). [a] One-way ANOVA with Bonferroni's post test with selected pairs was used to calculate the differences to the medium control ($P<0·05$).

faecal SCFA production is in the order of acetate > propionate > butyrate in a molar ratio of approximately 60:20:20 to 60:25:15, respectively[31,32]. Total SCFA concentrations have been found to range from 49 to 147 mM depending on the consumed diet as well as the region of the colon[33]. In the present study, the total SCFA concentrations increased after both fermentation series of the wheat samples from 47 and 45 mM to 132 and 128 mM, respectively, as well as after the fermentation of Synergy1® from 45 to 118 mM. Therefore, the detected values are comparable to physiological concentrations. These results are in line with previous studies using a simple *in vitro* fermentation model according to Barry *et al.*[9], in which SCFA concentrations increased two- to threefold compared with the faecal control after the fermentation of arabinoxylans[5] and Synergy1®[34].

In the present study, the statistical evaluation of the complex fermentation model II demonstrated the reproducibility by a comparable increase of total SCFA and butyrate after each fermentation (n 3) of Synergy1®. Furthermore, in other studies using the introduced *in vitro* fermentation II to digest a number of different substrates, namely wwf, wheat bran, wheat aleurone[20] as well as of different wheat and rye breads (our unpublished results) and nuts (our unpublished results), a comparable increase in the amounts of SCFA was detected. In addition, the reproducibility of the underlying *in vitro* fermentation simulating the colon was approved in a European interlaboratory study during the estimation of fermentability of dietary fibre sources by Barry *et al.*[9]. Here, in most cases, very similar

Table 3. Concentrations and molar ratios of SCFA as well as concentrations of bile acids (cholic (CA) and deoxycholic acid (DCA)) in fermentation supernatants (fs) of the faecal control (blank) and Synergy1® after fermentation II using 0·6 g/l ox gall*
(Mean values and standard deviations, n 3)

	fs blank		fs Synergy1®	
	Mean	SD	Mean	SD
SCFA (mM)				
Acetate	16·71	4·39	43·23[(b)]	18·52
Propionate	7·08	4·39	12·50	9·23
Butyrate	6·65	2·52	27·28[b]	2·64
Total SCFA (mM)	30·44	30·44	83·01[(b)]	30·35
Bile acids (μM)				
CA	0·36	0·12	0·27	0·19
DCA	36·06	10·27	2·23[b]	2·07

* Student's t test was used to calculate the differences to the fs blank (b: $P<0·05$). Parentheses denote the effect varying by trend ($P<0·10$).

results regarding SCFA production were found during a ring test, even though some interlaboratory differences of unknown origin remained.

The ratio of SCFA shifted slightly from acetate to propionate and butyrate (fermentation I) or to butyrate only (fermentation II) during the bacterial fermentation of wwf. In addition, fermentation (series II) of Synergy1® showed a shift towards butyrate which was comparable to wwf II. A particular interest in butyrate has arisen because of its potential preventive role against colon cancer[35]. Butyrate can have numerous biological effects, such as inducing apoptosis in tumour cells[36] and protecting cells from genotoxic insults by elevating phase II detoxification[37,38]. Therefore, the high production of SCFA *in vitro* is possibly an indication of protective effects by the diet.

Interestingly, the physiological amount of propionate constituting 20–25 % of the total SCFA amount clearly exceeded during fermentation I, whereas the concentration after fermentation II was still physiological[31,32]. Zampa *et al.*[39] showed that after the fermentation of xylo-oligosaccharides, propionate concentrations were high after 6 d of fermentation and then decreased, while butyrate started with low values and then increased to its highest values. During the first 6 d, the primary bile acid CA was converted into the corresponding secondary bile acid DCA with a conversion rate of more than 90 %. Additionally, it was observed that primary bile acid metabolism and the production of butyrate are inversely related during fermentation. Therefore, our observed high-propionate and low-butyrate proportions could possibly be a result of a reduced growth of butyrate-producing bacteria caused by the high-bile acid concentrations used in fermentation I.

Bile acid production

In general, our investigations showed that the fermentation of wwf and Synergy1® resulted in low levels of the secondary bile acid DCA, produced by bacterial conversion of primary bile acids. DCA has shown tumour-promoting properties by acting directly on the mucosa or by promoting the effects of carcinogenic substances present in the intestine[39]. Thus, the reduction of DCA can be regarded as positive in terms of colon cancer prevention. Besides reducing the solubility of bile acids, the lower pH can also reduce the activity of 7α-dehydroxylases, which are responsible for the conversion of primary to secondary bile acids[40]. Additionally, DCA may bind to dietary fibre, as has been demonstrated for CA and chenodeoxycholic acid by Elhardallou[41] who showed the binding of these bile acids by a fibre-rich fraction of different legumes. These results suggest that the dietary fibre sources wwf and Synergy1® may reduce the amounts of carcinogenic DCA in the colon. This finding is in line with the studies of our group using different sources of dietary fibres[34,42].

Effects on cell number

Several *in vitro* studies have indicated that SCFA, especially butyrate, reduce the growth of transformed colon cells[6,34,42], a parameter of secondary chemoprevention[43]. The generated fs used in the present study also led to an efficient reduction of the number of HT29 cells, whereas fs blank I exhibited the strongest effect. A synthetic mixture of CA and DCA of the corresponding complex fs blank I reduced the growth of HT29 cells significantly but less than the corresponding fs. Therefore, bile acids are apparently involved in growth reduction by fs blank I, but other factors in the complex fs seem to enhance these effects. A study by Shiraki *et al.*[44] supports these findings, because 100 μM-DCA reduced the proliferation rate of HT29. The change in ox gall concentration (0·6 g/l) used for the *in vitro* fermentation II resulted in fs wwf II, which was more effective in reducing cell growth than the corresponding blank II. Noteworthy, the less decreasing activity of fs blank II is comparable with other faecal controls, which were obtained with a single *in vitro* fermentation system and successfully used in further cell culture experiments[5,34]. Therefore, under these more physiological conditions comparable to a daily secretion of 20–30 g bile acids[21], the contained SCFA seem to be more important for the reduced cell growth than the contained reduced concentrations of bile acids (CA, 66–132 μM; DCA, 0·2–0·4 μM). Additionally, other ingredients of the fs such as antioxidant and antimutagenic compounds such as hydroxycinnamic acids might have contributed to the detected inhibition of cell growth, as was suggested by Beyer-Sehlmeyer *et al.*[6]. However, the high ox gall concentration (41·6 g/l) that was used for other *in vitro* models of digestion and for our fermentation I resulted in highly cytotoxic fermentation samples, and the contained bile acids apparently masked the effects of other, more physiological metabolites such as SCFA. A reduction of the ox gall to 0·6 g/l in our newly established model simulating the whole digestive tract is thus a critical parameter to use these samples in cell culture experiments. Therefore, the samples obtained using this optimised protocol can be used to study the effects of different foodstuffs on colon cell lines, and can therefore improve the outcome of *in vitro* studies.

Efficacy of in vitro *fermentation*

Predigestion of starch by α-amylase as well as the subsequent removal of glucose by dialysis is important for the following *in vitro* fermentation. It has been previously demonstrated that the fermentation of starch and glucose can result in high SCFA concentrations[45,46]. Therefore, these compounds can mask the results of dietary fibre fermentation if these compounds are not previously degraded and removed.

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In our experiments, an effective degradation of starch contained in wwf was demonstrated by increased concentrations of glucose in the retentate and dialysate. This reflects the hydrolysis of starch by added α-amylase and is in accordance with the results of Aura *et al.*[19]. In addition, this confirmed the subsequent removal of glucose by dialysis. Furthermore, the detection of the majority of the inserted bile acids in the dialysate demonstrated the efficiency of the dialysis. These results thus indicate that dialysis is an effective step to simulate absorption of small molecules occurring in the small intestine. But it has also to be kept in mind that the presented model is only an approach to physiological conditions. A complete physiological and selective absorption is limited by the absence of brush border enzymes. This is, however, a limitation that our model shares with all other available models simulating human digestion *in vitro*.

In conclusion, we introduce a modified *in vitro* digestion system that mimics the whole GIT. The samples obtained with this simulation can be used in cell culture experiments to study the effects of foodstuffs on colon cells. The reduction of high ox gall concentrations used in other models diminished cytotoxic side effects and enables an unmasked analysis of the effects of fermentation metabolites on cells. The fermentation of two different dietary fibre sources, namely wwf and Synergy1® (inulin enriched with oligofructose) using the presented system, resulted in the production of potentially chemopreventive metabolites such as SCFA, on the one hand, and a reduction of the tumour-promoting secondary bile acid DCA, on the other hand. Therefore, the designed protocol can successfully be used to study the effects of different nutrients and foodstuffs on colon cancer prevention and general markers of gut health in human colon cell cultures. Thus, the described *in vitro* fermentation model has already proved to be successful in subsequent investigations studying the chemopreventive effects (e.g. induction of apoptosis, differentiation and detoxification) of different fermented wheat sources[20,47–49].

Acknowledgements

The authors would like to thank the Federal Ministry of Education and Research, Germany (BMBF 0313829A) for funding. K. S., A. B., D. S. and M. G. designed the research and wrote the manuscript. K. S. and A. B. prepared the synthetic mixtures and performed the *in vitro* fermentation as well as the experiments on cell number measurements. K. S., G. B.-W. and U. O. performed the analysis of SCFA and bile acids and wrote the part describing the corresponding methods. J. H. and M. L. performed the analysis of glucose. N. W. provided wwf. We gratefully acknowledge the excellent technical assistance by Ms Esther Woschee. The authors have no conflicts of interest.

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