

## Mixtures of SCFA, composed according to physiologically available concentrations in the gut lumen, modulate histone acetylation in human HT29 colon cancer cells

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Intake of fibre has beneficial properties on gut health. Butyrate, a product of bacterial gut fermentation, is thought to contribute to positive effects by retarding growth and enhancing apoptosis of tumour cells. One mechanism is seen in its capacity to modulate histone acetylation and thereby transcriptional activity of genes. Next to butyrate, propionate and acetate are also major products of gut fermentation and together they may exert different potencies of cellular effects than butyrate alone. Since virtually nothing is known on combination effects by SCFA mixtures, here we had the aim to assess how physiological relevant concentrations and mixtures of SCFA modulate histone acetylation in human colon cells. HT29 colon cancer cells were incubated with mixtures of butyrate, acetate and propionate and with the individual compounds as controls. Histone acetylation was determined with acid-urea gel electrophoresis and immunoblotting. Acetylated histones slowly increased over 24 h and persisted up to 72 h in butyrate-treated HT29 cells. Butyrate (5–40 mM) and propionate (20–40 mM) enhanced histone acetylation significantly after 24 h incubation, whereas acetate (2.5–80 mM) was ineffective. Mixtures of these SCFA also modulated histone acetylation, mainly due to additive effects of butyrate and propionate, but not due to acetate. In conclusion, physiological concentrations of propionate together with butyrate could have more profound biological activities than generally assumed. Together, these SCFA could possibly mediate important processes related to an altered transcriptional gene activation and thus contribute to biological effects possibly related to cancer progression or prevention.

### SCFA: Histone acetylation: Colon tumour cells

Epidemiological studies support colon cancer-preventing activities of fruits, legumes and cereals containing fibre (Peters *et al.* 2003; Bingham *et al.* 2005). It has been implied that SCFA, produced in the gut lumen as a consequence of microbial fibre fermentation, are important in relation to fibre intake and colon cancer prevention (McIntyre *et al.* 1993). SCFA, and butyrate in particular, are energy sources for the colonic mucosal enterocytes. Moreover, butyrate seems to be of essential importance for the metabolic welfare of normal intestinal epithelia where it prevents apoptosis and subsequent mucosal atrophy (Wächterhäuser & Stein, 2000; Klampfer *et al.* 2003). Opposed to this, in colon carcinoma cells butyrate inhibits proliferation, causes differentiation and induces apoptosis at low concentrations (Barnard & Warwick, 1993; Scheppach, 1994; Hague & Paraskeva, 1995). Additionally, butyrate protects human colon cells from DNA damage (Abrahamse *et al.* 1999; Ebert *et al.* 2001) and induces members of the glutathione S-transferase biotransformation genes (Ebert *et al.* 2003; Pool-Zobel *et al.* 2005). An important mechanism by which butyrate causes biological effects in colon carcinoma cells has been proposed to be the hyperacetylation of H3 and H4 core histones by inhibiting histone deacetylases (HDAC) (Hinnebusch *et al.* 2002).

Histone acetylation influences transcription by loosening histone-DNA contacts, thus making the DNA accessible for transcription factors. Imbalance in histone acetylation can lead to transcriptional dysregulation and silencing of genes that are involved in control of cell-cycle progression, differentiation, apoptosis and cancer development (Marks *et al.* 2001). For instance, butyrate may mediate the reactivation of cell cycle arrest caused by the silenced gene p21<sup>WAF1/CIP1</sup> by acetylation of histones in its gene promoter (Archer *et al.* 1998; Sambucetti *et al.* 1999). Additional genes, also critical for cancer progression, were identified as inducible by histone acetylation of the promoter region (Iacomino *et al.* 2001; Ragione *et al.* 2001). The main SCFA, acetate and propionate, which also occur in the gut, have not been well investigated. Also, to our knowledge there are no reports available on the effects on histone acetylation of mixtures of SCFA, as they are formed in the gut lumen.

In contrast, the effects of butyrate are well characterized. A recent study on HT29 has shown that butyrate most effectively (2.2-fold) induced total histone acetylation at a concentration of 5 mM, followed by valerate (5 mM) (1.6-fold) and propionate (5 mM) (1.4-fold). Acetate (5 mM) did not enhance histone acetylation (Hinnebusch *et al.* 2002). Concentrations of 5 mM have

**Abbreviations:** HDAC, histone deacetylases; TSA, trichostatin A.

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been studied most frequently (Cousens *et al.* 1979; Siavoshian *et al.* 2000; Hinnebusch *et al.* 2002). In order to evaluate the biological impact of SCFA as found in the human gut, however, investigations of physiologically relevant concentrations are important. The three main SCFA, butyrate, propionate and acetate, can be found in the gut in considerably higher mM concentrations. Therefore, effects of SCFA on histone acetylation should be explored using the amounts expected to occur in the human gut lumen after ingestion of dietary fibre. These concentrations are 40–80 mM, 10–25 mM and 10–20 mM for acetate, propionate and butyrate, respectively (Schröder & Stein, 1997; Alles *et al.* 1999; Jenkins *et al.* 1999; Topping & Clifton, 2001). The relative molar proportions range from 50–65% for acetate, from 10–25% for propionate and from 10–25% for butyrate, depending on the fibre consumed (Schröder & Stein, 1997; Alles *et al.* 1999; Jenkins *et al.* 1999; Topping & Clifton, 2001).

The present study had the aim of investigating mixtures of SCFA, in concentrations as they occur in the gut lumen, on histone H4 acetylation in human colon cancer cells HT29. We also investigated the effects of the compounds individually, namely, acetate (2.5–80 mM), propionate (2.5–40 mM), butyrate (2.5–40 mM). The concentration ranges were larger than had been investigated before. The mixtures were composed at molar ratios of 69:16:15, 75:11:14 and 43:24:33 for acetate, propionate and butyrate, respectively. The compositions of these mixtures were based on the molar ratios found in *in vitro* fermentation samples obtained with human slurries and dietary fibre sources (Beyer-Sehlmeyer *et al.* 2003). The last mentioned mixture, particularly, reflects the fermentation of dietary fibres that are more beneficial to gut health and integrity. A predominant property of putative beneficial mixtures is that they contain relatively high butyrate concentrations (Topping & Clifton, 2001). Together, these studies were expected to provide novel information on whether the mixtures of SCFA were additive, inhibitory or even synergistic for modulating histone acetylation in comparison with each individual compound on its own.

## Materials and methods

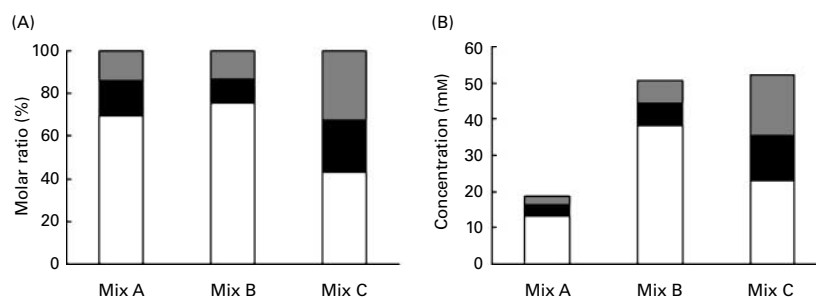
### Cell culture conditions

The human colon carcinoma cell line HT29, established in 1964 (Fogh & Trempe, 1975), was obtained from the

American Tissue Culture Collection (Rockville, MD, USA). The cells were maintained in Dulbecco's Modified Eagle Medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Invitrogen) and 1% penicillin–streptomycin (Life Technologies, Karlsruhe, Germany), grown in a humidified incubator (5% CO<sub>2</sub>; 95% humidity; 37°C) and harvested with trypsin–versene solution (2.5%; 1:5000; Invitrogen). Passages 26 to 53 were used for the experiments presented here. Viability was always over 85%.

### Treatment of HT29 cells with SCFA and trichostatin A

Stock solutions of Na-butyrate, Na-acetate (Merck, Darmstadt, Germany) and Na-propionate (Fisher Scientific GmbH, Schwerte, Germany) (200 mM each) were prepared in Dulbecco's Modified Eagle Medium and frozen for storage until use (–20°C). Similarly, SCFA mixtures were frozen as 50 × stock solutions of the three main SCFA, acetate, propionate and butyrate. To yield appropriate concentrations in the cell culture medium, these mixtures were prepared as a 1 × solution before each experiment. The '100% mixture' A contained 18.8 mM-SCFA at the ratio of 69:16:15 (acetate:propionate:butyrate); mixture B contained 50.8 mM-SCFA at the ratio of 75:11:14; mixture C was composed of 52.4 mM-SCFA at the ratio of 43:24:33 (see also Fig. 1). For assessing concentration-related effects of SCFA and mixtures, 3 × 10<sup>6</sup> cells were seeded for 24 h in six-well plates (Fisher Scientific GmbH) and treated for an additional 24 h with butyrate (2.5–40 mM), propionate (2.5–40 mM), acetate (2.5–80 mM) and SCFA mixtures A, B and C (100% and at 10% dilutions) or medium alone (medium control). Trichostatin A (TSA; Sigma-Aldrich, Taufkirchen, Germany) was used as a positive control since it is an effective inducer of histone acetylation (Chen & Townes, 2000). TSA was diluted in ethanol to yield a 3.3 mM stock solution and then stored frozen (–20°C) until further use. For comparing kinetics of butyrate and TSA-mediated effects on histone acetylation, HT29 cells were grown in 75 ml flasks (Fisher Scientific GmbH) to 90–100% confluence within 5 d. Butyrate (5 mM) or TSA (3.3 μM) were added for different time periods (30 min–72 h). In these experiments, medium with ethanol (0.1%) served as the medium control. After incubation, both SCFA and TSA-treated cells were harvested and washed with PBS. The number of cells and cell viability



**Fig. 1.** Relative molar concentrations (A) and absolute concentrations (B) of acetate (□), propionate (■) and butyrate (▒) in the mixtures A, B and C. The mixtures are composed to simulate SCFA pattern as obtained after fibre fermentation using human gut flora *in vitro*. Mixture A corresponds to a low fermentable fibre (watercress). Mixtures B and C are composed to simulate a high fermentable fibre with a lower and a high ratio of butyrate + propionate:acetate, as described for inulin and linseed flour (Beyer-Sehlmeyer *et al.* 2003).

was determined. The suspension was centrifuged and the cell pellets were stored at  $-20^{\circ}\text{C}$  until histone extraction.

#### Histone extraction

Cell pellets (approximately  $8 \times 10^6$  cells) were homogenized twice with a pellet-pistill (Fisher Scientific GmbH) in 1 ml lysis buffer (1.2 g/l Tris; 5.2 g/l  $\text{Na}_2\text{SO}_4$ ; 2 g/l  $\text{MgCl}_2$  ( $\times 6 \text{ H}_2\text{O}$ ); 86 g/l sucrose; 0.24 g/l pefabloc; 1 % Triton-X-100 (v/v); 1.54 g/l dithiothreitol; pH 6.5) and the homogenate was centrifuged (1000 g; 10 min,  $4^{\circ}\text{C}$ ). The supernatant fractions (cytosol) were discarded and the nuclear pellets were washed with PBS. Acid soluble proteins (histones) were extracted in 200  $\mu\text{l}$  extraction buffer (3.46 g/l Tris; 13.8 g/l  $\text{Na}_2\text{EDTA}$ ; 0.24 g/l pefabloc; 1.54 g/l dithiothreitol; pH 7.4) with 1.1 % (v/v) concentrated  $\text{H}_2\text{SO}_4$ . After 2 h extraction, tubes were centrifuged (11 000 g; 10 min,  $4^{\circ}\text{C}$ ) and their supernatant fractions were precipitated with acetone overnight ( $-20^{\circ}\text{C}$ ). On the next day, tubes were again centrifuged (11 000 g, 20 min,  $4^{\circ}\text{C}$ ), supernatant fractions were discarded and the pellets containing the histones (H1; H2A; H2B; H3; H4) were allowed to dry at room temperature (Cousens *et al.* 1979; Ajiro *et al.* 1981). Histones were diluted in distilled water and frozen at  $-20^{\circ}\text{C}$ . Protein content was determined with an assay according to Bradford (1976) using bovine serum albumin as standard protein.

#### Acid-urea gel electrophoresis

The method of acid-urea gel electrophoresis is frequently used to determine total histone acetylation (O'Neil *et al.* 1999; Chen & Townes, 2000; Pender *et al.* 2000). Acid-urea gel polymerization (20 % bis/acrylamide; 5 % acetic acid; 0.36 g/l urea;  $20 \times 20/1.5$  mm) was performed overnight. Gels were then conditioned for approximately 2 h pre-electrophoresis (cathodic PAGE: 5 % acetic acid; 400 V;  $13^{\circ}\text{C}$ ) followed by a 1-h run with scavenge buffer (5 % (v/v) acetic acid; 0.36 g/ml urea; 0.068 g/ml mercaptoethylamine; 10 % (v/v) glycerol; methyl green). Frozen histones were thawed and heated at  $95^{\circ}\text{C}$  for 5 min. The volume with a total of 12  $\mu\text{g}$  histones (from approximately  $1 \times 10^6$  cells) was diluted with the equal volume of 2  $\times$  acid-urea-loading buffer (5 % (v/v) acetic acid; 0.36 g/ml urea; 20 % (v/v) glycerol; 0.003 g/ml dithiothreitol; methyl green). The samples were loaded onto the gel together with a standard solution composed of core histones (Upstate Biotechnology, New York, USA). Electrophoresis was performed with 5 % (v/v) acetic acid electrophoresis buffer at 400 V and  $13^{\circ}\text{C}$  for 24 h. Then gels were washed with bidest and stained with Coomassie blue (Coomassie Brilliant Blue R-250; Sigma-Aldrich). Five bands representing the different histone H4 acetylation variants were analysed densitometrically using 'Fluor S Multi Imager' (BioRad, München, Germany). Data were expressed as the percentage of band signals for deacetylated ( $\text{H}_{4_0}$ ) or acetylated (sum of  $\text{H}_{4_1}$ ,  $\text{H}_{4_2}$ ,  $\text{H}_{4_3}$ ,  $\text{H}_{4_4}$ ) H4 variants compared with the sum of all five bands of histone H4 (whole H4) (optical density units (%)). Non-detectable bands were set to equal zero. ( $\text{H}_{4_1}$  1-fold;  $\text{H}_{4_2}$  2-fold;  $\text{H}_{4_3}$  3-fold;  $\text{H}_{4_4}$  4-fold acetylated H4.)

#### Histone immunoblotting

Frozen histones were thawed and heated at  $95^{\circ}\text{C}$  for 5 min. Histones (12  $\mu\text{g}$ ) were diluted with the equal volume of 2  $\times$  loading buffer (0.03 g/ml Tris; 4 % (w/v) SDS; 20 % (v/v) glycerol; 0.003 g/ml dithiothreitol; bromphenolblue; pH 6.8). The mixture was loaded onto a  $10 \times 10$  cm bis/acrylamide gel (5 % stacking gel; 15 % resolving gel) together with a molecular weight standard (low range; Biomol, Hamburg, Germany). After electrophoresis (120 V; 45 min), the gel was blotted on a polyvinylidene fluoride (Schleicher & Schuell, Dassel, Germany) membrane (1 mA/cm<sup>2</sup>; 2 h). The blot was blocked with 5 % milk protein in Tris-buffered saline (0.1 % v/v Tween, 20) for 1 h and then incubated with the primary H4 antibody (1 : 1000; 5 % bovine serum albumin in Tris-buffered saline Tween) (Roche, Karlsruhe, Germany) overnight. Afterwards, blots were washed with Tris-buffered saline Tween, incubated with the secondary rabbit-alkaline phosphatase antibody (1 : 1000; 5 % milk protein; Dako Deutschland GmbH, Hamburg Germany), and chemifluorescence signals for whole H4 (as the control for equal sample loading) were determined using enhanced chemifluorescence as the substrate (Amersham, Freiburg, Germany). Optical density units (count/mm<sup>2</sup>) were measured using a Fluor S Multi Imager (BioRad). For determining H4-acetylation, blots were stripped and all the aforementioned steps were repeated using the acetylated H4 antibody (Upstate Biotechnology). For comparing degrees of acetylation, the resulting fluorescence signal of acetylated H4 antibody was corrected for the signal of H4 antibody (internal control for equal sample loading) and normalized relative to the medium or 5 mM-butyrate positive control (equal to 1).

#### Statistical evaluation

The GraphPad Prism software Version 4 (GraphPad Software Inc., San Diego, CA USA) was used to calculate a one-way ANOVA with Tukey's post test. Data of at least three experiments were evaluated to establish two-sided significance levels of independently reproduced determinations.

## Results

#### Time response kinetics of butyrate on histone H4 acetylation in HT29 cells

Before investigating the mixtures, first detailed studies on kinetics of butyrate-mediated effects were performed to establish assay conditions and to determine the sensitivity of the cell line. TSA, which is known for its pronounced histone acetylating activity (Chen & Townes, 2000), was used as a positive control. Table 1 shows that butyrate mediated (5 mM) histone acetylation increased slowly, as is apparent by the significant difference between deacetylated and acetylated H4, beginning at 4 h ( $P < 0.01$ ) up to 24 h ( $P < 0.001$ ). This plateau persisted for up to 72 h ( $P < 0.05$ ). In contrast to butyrate, 3.3  $\mu\text{M}$ -TSA immediately induced histone H4 acetylation as early as after 0.5 h incubation ( $P < 0.001$ ), with a maximum induction after 4 h ( $P < 0.001$ ). Subsequently, TSA acetylated histone H4 decreased to corresponding control levels after 24 h. No effect was observed in the medium control. Based on these studies we performed our concentration finding studies for SCFA using treatment times up to 24 h.

**Table 1.** Kinetics of histone acetylation after treatment with butyrate and trichostatin A (TSA)††  
(Mean values and standard deviations for three to four experiments)

Treatment	Time of incubation	Deacetylated H4		Acetylated H4	
		Mean	SD	Mean	SD
Medium control	30 min	39.0	6.6	61.0	6.6
	4 h	43.4	7.4	56.6	7.4
	6 h	42.3	6.2	57.7	6.2
	10 h	39.0	7.8	61.1	7.8
	24 h	36.9	7.7	63.1	7.7
	72 h	42.6	5.5	57.4	5.5
Butyrate (5 mm)	30 min	34.9	12.6	65.1	12.6
	4 h	29.6	13.2	70.4**	13.2
	6 h	28.0	14.8	72.0**	14.8
	10 h	27.3	14.2	72.8**	14.2
	24 h	25.7	16.9	74.3***	16.9
	72 h	27.5	6.3	72.5*	6.3
TSA (3.3 µm)	30 min	20.1	13.6	79.9***	13.6
	4 h	15.7	12.4	84.3***	12.4
	6 h	21.1	18.1	79.0***	18.1
	10 h	28.2	17.0	71.8***	17.0
	24 h	36.0	11.1	64.0	11.1
	72 h	44.2	7.2	55.8	7.2

One-way ANOVA with Tukey's multiple comparison test were used to calculate the differences between the signals for acetylated histone H4 (sum of H4<sub>1</sub>, H4<sub>2</sub>, H4<sub>3</sub>, H4<sub>4</sub>) to deacetylated histone H4 (H4<sub>0</sub>): \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001.

† After acid-urea gel electrophoresis, gels were stained with Coomassie blue, and bands for H4 variants were analysed densitometrically.

†† For details of procedures, see p. 805.

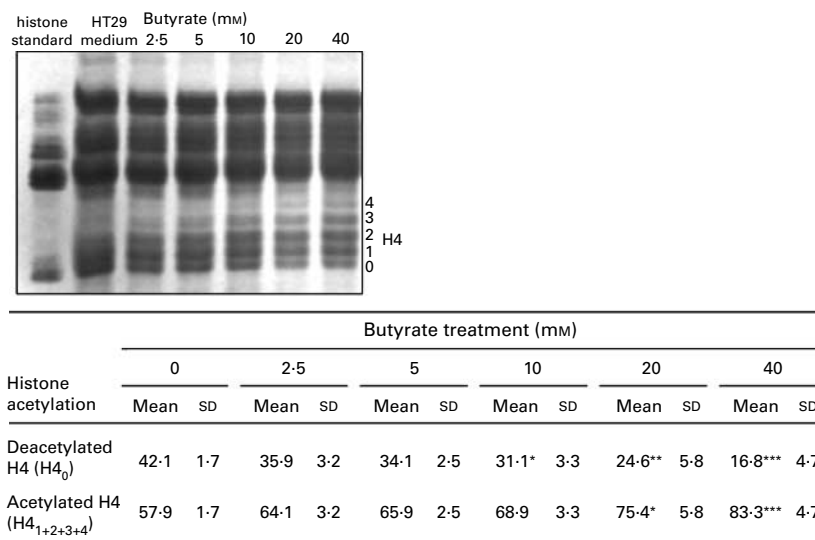
*Concentration effect relationships of individual SCFA: butyrate*

Fig. 2 shows a sample gel and the effects of 2.5–40 mM-butyr-ate on histone acetylation determined in three independently

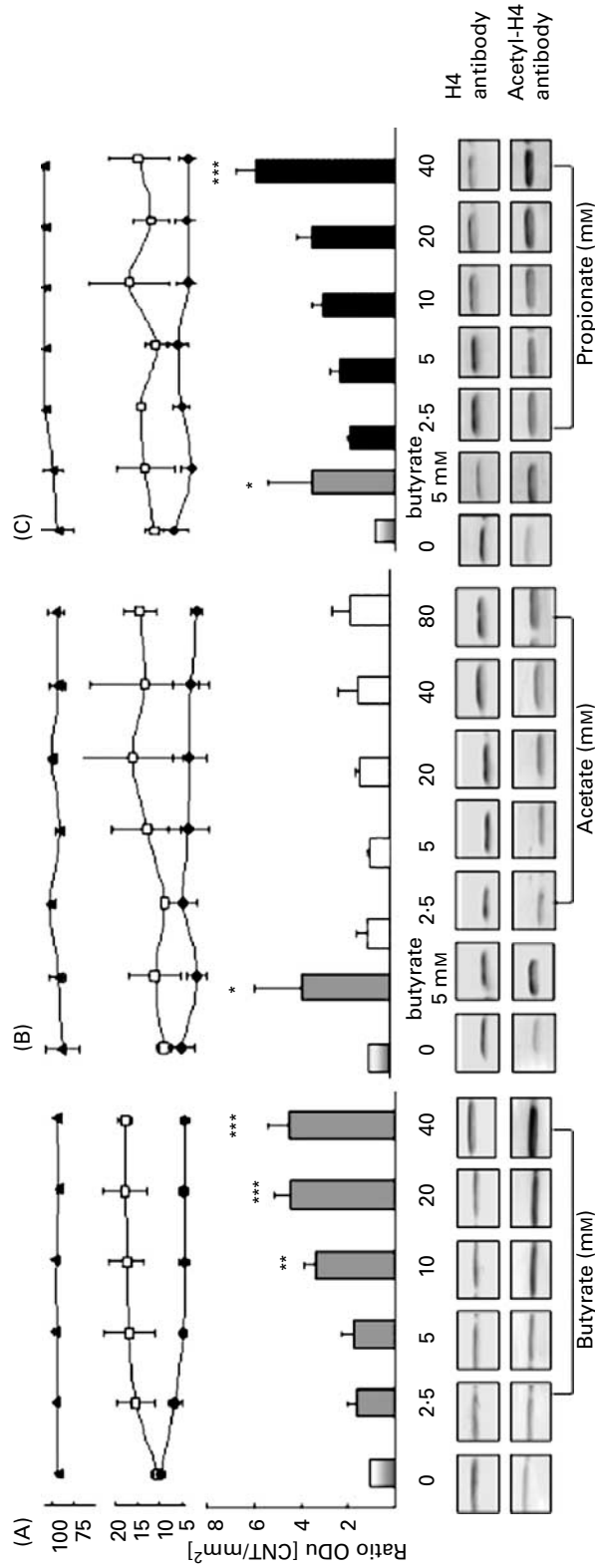
reproduced experiments. We found a concentration-related increase in band intensities of 1- to 4-fold acetylated H4 variants (H4<sub>1</sub>, H4<sub>2</sub>, H4<sub>3</sub>, H4<sub>4</sub>) and a decrease of deacetylated H4 (H4<sub>0</sub>) after 24 h treatment. Changes within histone H4 variants were characterized as a step-by-step modulation in the degree of acetylation. Compared with the medium control, the effects were significant after incubation with 10 mM (*P*<0.05), 20 mM (*P*<0.01; *P*<0.05) and 40 mM (*P*<0.001) butyrate. Important changes were the decrease of the lowest band (H4<sub>0</sub>) with simultaneous increases of the third and fourth bands (H4<sub>2</sub>, H4<sub>3</sub>). Results were confirmed using the specific method of immunoblotting, as shown in Fig. 3(A). Histone H4 acetylation was significantly induced at concentrations of 10 mM (*P*<0.01), 20 mM (*P*<0.001) and 40 mM (*P*<0.001) butyrate. Additionally, there was a trend (not significant) for an increased yield of histone proteins after butyrate treatment (1.7-fold). Cell number was decreased to approximately 50% by 10–40 mM-butyr-ate treatment, however, without any effect on cell viability. Thus, histone acetylation was significantly modulated after 24 h treatment with butyrate at a concentration that resulted in a reduced cell number but not cell viability.

*Acetate and propionate*

We investigated acetate (2.5, 5, 20, 40 and 80 mM) and propionate (2.5, 5, 10, 20 and 40 mM) at concentrations that resembled the physiologically available ranges that are expected to occur in the gut lumen after ingestion of dietary fibre. In these experiments, butyrate (5 mM) served as the positive control. In comparison with the medium control, no induction of histone acetylation was observed for acetate up to 80 mM (Fig. 3(B)). Propionate, however, significantly modulated histone acetylation at concentrations of 20 mM (*P*<0.05) and 40 mM (*P*<0.001) (Fig. 3(C)). To enable a better comparison of inducibility of histone acetylation



**Fig. 2.** Changes of deacetylated (0) and acetylated (1, 2, 3, and 4) histone H4 after 24 h treatment with butyrate. After acid-urea gel electrophoresis, gels were stained with Coomassie blue and bands for H4 variants were analysed densitometrically. Data were expressed as the percentage of band signals compared with the sum of all five bands for histone H4 (whole H4). One-way ANOVA with Tukey's multiple comparison test were used to calculate the differences between deacetylated (H4<sub>0</sub>) as well as acetylated histone H4 (sum of H4<sub>1</sub>, H4<sub>2</sub>, H4<sub>3</sub>, H4<sub>4</sub>) variants after treatment, to corresponding medium controls. Values are means and standard deviations for three independent experiments: \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001). For details of procedures, see p. 805.



**Fig. 3.** Yield of acetylated H4. Histones after 24 h incubation with physiological concentrations of butyrate (■), acetate (□) and propionate (▨) using immunoblot. Samples were loaded onto a 15% bis/acrylamide mini-gel, blotted on a polyvinylidene fluoride membrane and incubated with H4 antibody and acetyl-H4 antibody. Signal of acetylated H4 antibody was corrected for signal of H4 antibody, and the increase of acetylation was expressed as a ratio of the optical density unit (ODu) (count CNT/mm<sup>2</sup>) in the treated group relative to the medium control. Also shown are the results on determination of cell viability (%; -▲-), content of histone proteins (µg per million cells; -□-) and total cell number (million; -◆-). The data are presented as mean values with their standard deviations for three independent experiments. One-way ANOVA with Tukey's multiple comparison test were used to calculate the differences between the treated groups to corresponding medium controls: \**P*< 0.05; \*\**P*< 0.01; \*\*\**P*< 0.001; \*\*\*\**P*< 0.0001. For details of procedures, see p. 805.

during different incubation conditions, we evaluated the relative acetylation levels by normalizing the values to the positive control (5 mM-butyrate). These analyses revealed that 10 and 20 mM-propionate had approximately half of the acetylation-inducing capacity of butyrate, whereas 40 mM-propionate was as effective as 40 mM-butyrate (data not shown). At the highest SCFA concentrations, the cell numbers were decreased by approximately 40 %, without any effect on viability or histone-protein content.

#### *Effects of SCFA mixtures containing butyrate, propionate and acetate on histone acetylation*

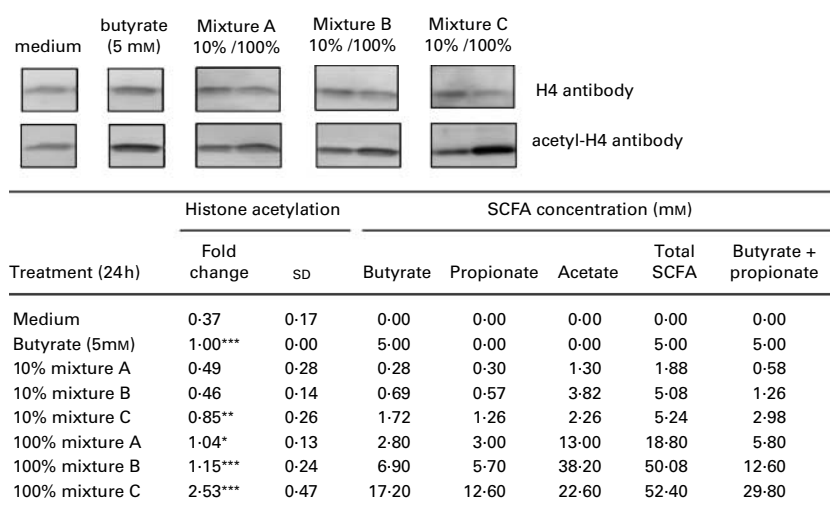
Next we determined effects of mixtures of butyrate, propionate and acetate in various relative concentrations. The compositions of the 100 % mixtures (and their 10 % dilutions) were based on the observed SCFA concentrations of various fermentation samples obtained from different fibre sources using human faecal slurries *in vitro* (Beyer-Sehlmeyer *et al.* 2003). They mimicked the relative concentrations probably occurring in the gut (Barry *et al.* 1995; Wächterhäuser & Stein, 2000). Molar ratios of acetate, propionate and butyrate of mixtures A and B are comparable with each other (69 : 16 : 15 *v.* 75 : 11 : 14), but mixture A contained approximately only one-third of the total SCFA amounts used to prepare mixture B (38.2, 5.7 and 6.9 mM, respectively). Opposed to this, mixture C contained much higher propionate and butyrate amounts (12.6 and 17.2 mM, respectively) with a molar ratio of 43 : 24 : 33. Its total SCFA concentration, however, was comparable to mixture B (Fig. 1). Again, butyrate (5 mM) was used as the positive control during these experiments. Fig. 4 shows that the different mixtures had different potencies of effectiveness. We normalized the results on the basis of the positive control (5 mM-butyrate), which was set to equal 1. Differences between medium and positive control were significant ( $P < 0.001$ ). Among the diluted mixtures at concentrations of 10 %, only mixture C, which had the highest

amount of butyrate and propionate (molar ratio of 43 : 24 : 33), induced histone acetylation significantly ( $P < 0.01$ ). However, all three undiluted mixtures (100 %) induced histone acetylation ( $P < 0.05$ ;  $P < 0.001$ ). Mixture A, at the 100 % concentration, induced histone acetylation to the same extent, as the 5 mM-butyrate positive control (1.04- *v.* 1.00-fold). At this amount, mixture A contains only 2.8 mM-butyrate, but 5.8 mM-butyrate + propionate. Induction by mixture B (1.15-fold) was less than by mixture C (2.53-fold). Again, not the total SCFA amounts (50.8 *v.* 52.4 mM), but rather more the sum of butyrate and propionate (12.6 *v.* 29.8 mM) seemed to be the important factor for induction of H4 histone acetylation. Results show also, when contained in mixtures of SCFA, butyrate was the most important factor for histone acetylation, followed by propionate, whereas acetate seemed to have no further effects. In addition, we found additive effects of butyrate and propionate reflecting the sum of their individual capacity to induce histone acetylation, but synergistic effects were not apparent.

#### Discussion

Acetylation of histones in the DNA of cancer cells has been associated with the reactivation or silencing of genes critical for cancer progression, differentiation and apoptosis (Sambucetti *et al.* 1999; Iacomino *et al.* 2001; Wu *et al.* 2001). The activation of genes by inhibiting histone deacetylators (HDAC) may be an important biological effect mediated by exogenous factors. Among different HDAC repressors, SCFA are of special interest since they occur naturally in the gut. The present study aimed to assess the importance of mixtures of SCFA for histone acetylation. For these studies we used concentrations as they are expected to occur in the gut lumen after ingestion of different dietary fibres.

Time response kinetics of histone acetylation by butyrate and TSA (a well-known HDAC-inhibitor) were compared in HT29 colon cancer cells that were treated for 0.5–72 h.



**Fig. 4.** Modulation of HT29 histone acetylation after incubation with different SCFA mixtures (10 % and 100 % of physiologically available concentrations) and butyrate as a positive control by immunoblotting. Signals for acetylated H4 were corrected for whole H4 and compared with 5 mM-butyrate. Mean values were significantly different from those for the other treatments (one-way ANOVA with Tukey's multiple comparison test): \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; different concentrations were separately analysed. Molar ratios of acetate:propionate:butyrate were: mixture A = 69 : 16 : 15; mixture B = 75 : 11 : 14; mixture C = 43 : 24 : 33. For details of procedures, see p. 805.

The observed different effects of TSA and butyrate on histone acetylation were probably due to the shorter half life of TSA, as had been hypothesized earlier (Siavoshian *et al.* 2000). Removal of the HDAC-inhibitor, TSA, resulted in rapid deacetylation of histones within 4 h (Chen & Townes, 2000). In contrast, the acetylation patterns by butyrate, as detected in the present study, may be a reflection of the kinetics of butyrate uptake by the cells, as we have reported earlier (Kautenburger *et al.* 2005). The studies have shown that approximately 15, 40 and 60% of the applied butyrate (2 mM) are consumed by HT29 cells after 24, 48 and 72 h treatment, respectively. The resulting slow, but persistent, activation of histone acetylation by butyrate consequently may be important for the cancer-protecting activity of butyrate in the gut. Thus, Wu *et al.* (2001) have shown that prolonged histone hyperacetylation is required for induction of differentiation, apoptosis and growth factor unresponsiveness in HT29 cells. Therefore, a continuous and repetitive fibre intake may produce a prolonged histone hyperacetylation, which may be of physiological importance.

Next, concentration effect responses of SCFA (butyrate, propionate and acetate) on histone acetylation were assessed in the genome of colon cells. For these experiments concentrations were chosen that reached those that have been shown to occur under physiological relevant conditions after ingestion of different types of dietary fibre (Knudsen *et al.* 1997; Alles *et al.* 1999; Wächterhäuser & Stein, 2000). This was a much larger SCFA concentration range than had been investigated before and resulted in an increase in histone acetylation at the higher butyrate and propionate concentrations. The effect was accompanied by growth suppression but not by a decreased viability of colon cells.

The major aim of the present study was to investigate the impact of SCFA as components of a mixture. These were made up to simulate the butyrate, propionate and acetate concentrations that had been analysed in various samples obtained by fermenting dietary fibre sources with human faecal slurries *in vitro* (Beyer-Sehlmeyer *et al.* 2003) and, therefore, possibly also occurring in the gut (Barry *et al.* 1995). Mixture A corresponds to fermentation of a low fermentable fibre such as that found in watercress (Beyer-Sehlmeyer *et al.* 2003). Mixture B resembled the relative SCFA contents after fermentation of inulin, kale or soya, whereas the composition of mixture C resembled the SCFA content after fermentation of linseed flour (Beyer-Sehlmeyer *et al.* 2003). Our previous studies on capacities of SCFA mixtures to modulate proliferation have shown additive effects of butyrate and propionate in suppressing HT29 growth (Beyer-Sehlmeyer *et al.* 2003). In the present study we show that there are also additive effects of propionate on induction of histone acetylation. It was recently reported that there are differences between the uptake of butyrate (55% 2 mM), acetate (59% 6 mM) and propionate (33% 2 mM) after 24 h incubation of intestinal epithelial goblet cells. This is connected to a repression of propionate uptake (down to 5%) by the other components of the SCFA mixture (Gaudier *et al.* 2004). However, this property did not seem to play a role for modulation of histone acetylation in our experiments. The present results rather more support the hypothesis that the fibre fermentation product, propionate, has additive effects on butyrate and thus plays a much more important role for histone acetylation in the gut than was assumed

previously. Moreover, whereas the majority of butyrate is used up by colonocytes as a source of energy, propionate could still be active when it reaches the liver, before it is further metabolized (Roberfroid, 2005).

The present results verify the importance of SCFA, in particular of butyrate and propionate, as inducers of histone acetylation, which is possibly associated with a modulated growth response of neoplastic or tumorigenic lesions in the gut. Propionate not only adds to butyrate's effect of suppressing growth, as shown previously, but also to butyrate's effect of modulating histone acetylation in human colon cells. Translated to the *in vivo* situation, the products of complex *in vitro* fibre fermentation, including butyrate and propionate, may contribute to suppressing agent activities *in vivo* resulting in the inhibition of tumour progression (Wattenberg, 1992; Johnson, 1995). It will be of interest in the future to determine effects on histone acetylation by additional fibre fermentation products, as they are formed in the gut. In light of the frequency of microneoplasia, which markedly increase with age (Heavey *et al.* 2004), the present findings are of relevance, since they possibly point to SCFA's potential to inhibit growth and survival of transformed colon cells and, thus, further progression. Now, it would be of interest to investigate in more depth how the observed effects by complex SCFA mixtures also affect gene expression and then to further identify involved genes.

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