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Apple Polyphenols and Products Formed in the Gut Differently Inhibit Survival of Human Cell Lines Derived from Colon Adenoma (LT97) and Carcinoma (HT29)

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Colorectal tumor risks could be reduced by polyphenol-rich diets that inhibit cell growth. Here, apple polyphenols were studied for effects on the survival of colon adenoma (LT97) and carcinoma-derived (HT29) cell lines. Three apple extracts (AEs) from harvest years 2002−2004 were isolated (AE02, AE03, and AE04) and fermented in vitro with human fecal flora. Extracts and fermentation products were analyzed for polyphenols with HPLC. The cells were treated with AEs (0−850 μg/mL) or fermented AEs (F-AEs, 0−9%), and survival was measured by DNA staining. All AEs contained high amounts of polyphenols (311−534 mg/g) and reduced cell survival (in LT97 > HT29). AE03 was most potent, possibly because it contained more quercetin compounds. Fermentation of AEs resulted in an increase of short chain fatty acids, and polyphenols were degraded. The F-AEs were ∼3-fold less bioactive than the corresponding AEs, pointing to a loss of chemoprotective properties through fermentation.

KEYWORDS: Antiproliferative activity; apple polyphenols; colon cancer chemoprevention; fermentation; colon cell line

INTRODUCTION

Epidemiological findings suggest that plant foods decrease colorectal tumor risks (*1*). This could be due to a number of different phytoprotectants, including polyphenolic flavonoids, which act chemopreventive by inhibiting the growth of tumor cells (*2*−*4*). It has also been shown that compounds such as flavonoids can affect processes that induce differentiation, cause apoptosis, and enhance anti-inflammatory responses in colorectal cancer cell lines (*5*−*7*). Apple flavonoids, such as phloridzin, quercetin, and (+)-catechin, have been shown to exert potent antiproliferative activities in several studies (*8*−*10*), and it has been shown that they are able to scavenge endogenous reactive oxygen species (ROS) (*11*). Other than acting as antioxidants (*12*) or scavenging carcinogens, the apple polyphenols may inhibit carcinogenesis by affecting molecular events in the initiation, promotion, and progression stages (*13*). Apples contain very high amounts of polyphenols (*14*), which vary depending on the variety (*15*). The total amount of polyphenols that can be extracted from 100 g of fresh apples ranges from 110 to 357 mg (*16*), and quercetin and its glycosides are the most abundant polyphenols found in apples (*17*).

Eberhardt et al. reported that an apple extract (AE) inhibited the proliferation of HepG2 liver tumor cells in vitro and suggested that these antiproliferative effects could have been due to the presence of a unique combination of phytochemicals in the apples in addition to ascorbic acid (*18*). We have performed similar studies with human colon carcinoma cells (HT29) and compared the effects of an AE, a synthetic AE (mixture of the major polyphenols that mimicked AE), and individual polyphenol compounds. It was shown that both mixtures significantly inhibited the growth of HT29 cells in a dose- and time-dependent manner (*10*). However, the effect differed between the test compounds (individual polyphenols < synthetic AE < natural AE). The findings thus suggested that complete mixtures of phytochemicals in fresh fruits were more antiproliferative in HT29 cells than the sum of individual ingredients when tested alone. Apples are a significant source of flavonoids in people's diet in the U.S. and Europe (*19*, *20*). The ability of the mammalian colon to absorb apple polyphenols has been shown in vivo and in vitro (*21*). About 0−33% of polyphenols reached the colon and were fermented by the gut bacteria (*22*). This means that both original apple polyphenols

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as well as metabolites formed physiologically in the gut are present in the lumen and theoretically may interact with the enterocytes.

Therefore, here we had the aim of investigating unaltered AEs, as well as their counterparts fermented by the human gut flora. Our expectation was that the fermented apple polyphenol extracts (F-AEs) should differ from the unfermented polyphenol extracts (AEs). This was hypothesized because certain key flavonoids in the fresh, unfermented state are bound to sugar moieties and thus occur as glycosides, galactosides, rhamnosides, etc. The fermentation process is expected to liberate the aglycones of the flavonoids by cleaving the flavonoid sugar complexes. Alternatively, it is known from the literature (*23*) that polyphenols can be metabolized to short chain fatty acids (SCFA) of which butyrate and propionate have been shown to inhibit the growth of HT29 cells (*24*). The key question to be solved here was therefore to determine if the fermentation process leads to an alteration of the biological activities.

Different AEs with different polyphenolic compositions were investigated together with their corresponding fermentation products produced by incubation of the AEs with human gut flora under anaerobic conditions. Polyphenolic compositions of AEs and F-AEs were compared. Also, the effects on proliferation were determined in the human colon adenocarcinoma cell line HT29, as this was previously shown to be a sensitive model of biological activity (*25*). We also determined effects in the LT97 colon adenoma cell line, which represents an early premalignant stage of tumor development (*26*).

MATERIALS AND METHODS

Preparation of the Extract Containing Apple Polyphenols. Clear apple juice was produced on an experimental scale. In compliance with the usual practice of apple juice production, we used a defined mixture of apple varieties consisting of Jonagold (20%), Topaz (25%), Bohnapfel (17.5%), Winterrambur (22.5%), and Bittenfelder (15%). This type of well-balanced mixture of cider and table apples is required to achieve adequate sensorial properties. Polyphenols from 100 L of the resulting apple juice were retained on a 5 L adsorber resin (XAD 16 HP, Rohm and Haas, Frankfurt, Germany) that was packed in a Pharmacia glass column (BPG 100, 100 cm × 10 cm). Water soluble juice ingredients like sugars, organic acids, and minerals were removed by washing using six bed volumes of distilled water. Polyphenols were eluted with three bed volumes of ethanol (96%). The ethanolic fraction was gently concentrated by evaporation and transferred to an aqueous solution that was then freeze-dried (*27*).

Quantification of Polyphenols in AEs. A solution of the apple polyphenol extracts was prepared to yield a concentration of 1 g/L in 20% methanol. The solution was filtered (0.2 μm), and 20 μL was injected into an HP1090HPLC system equipped with a photodiode array detector (Hewlett-Packard, Palo Alto, CA). A Phenomenex aqua column (250 mm × 4 mm, Phenomenex, Aschaffenburg, Germany) was used at ambient temperature. Gradient elution was performed using an acetonitrile (ACN)/phosphoric acid gradient. Detection wavelengths were 280 nm for flavonoids, 320 nm for phenolcarbonic acids, and 360 nm for quercetin derivatives. Quantitation was carried out using peak areas from external calibrations with standard solutions (*27*). After isolation, the extracts were stored in a dark place at ambient temperature for 6 months, and this time, there were no significant differences of the polyphenols. Moreover, the proliferation assay and fermentation experiments were performed within 2 months after production of AEs. Also, the proliferation assay for F-AEs was done after only 1 month of storage (−80 °C).

Preparation of the Fermented Apple Polyphenol Extracts. All fermentations were conducted in vitro under anaerobic conditions (80% nitrogen, 10% carbon dioxide, and 10% hydrogen at 37 °C), basically according to described procedures (*28*). A mixture of fresh human feces from three healthy volunteers who had given their informed consent was prepared as a bacterial source. These were used to ferment the reconstituted AEs (AE02, AE03, and AE04). The volunteers consumed their normal, non-vegetarian diet without any restrictions. The study was approved by the Ethical Committee of the Friedrich-Schiller-University Jena.

The fecal samples were immediately weighed and filled into one homogenizing bag. Potassium phosphate buffer (0.1 M, pH 7.0) was added (5:1 v/w), and the mixture was homogenized thoroughly in a Stomacher 400 (Seward, Worthing, UK). From the fecal homogenate, 40 mL aliquots were filled into 500 mL glass bottles. Apple polyphenol samples were dissolved in anaerobic potassium phosphate buffer to provide 20 g/L fermentable substances. A total of 40 mL of each polyphenol solution was added to separate bottles to obtain a final AE content of 10 g/L and a fecal suspension of 10% as recommended by Barry et al. (*29*). As a negative control (blank), potassium phosphate buffer was added to one bottle instead of apple polyphenols. Anaerobic conditions in the glass bottles were achieved by removing the 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 (15 cycles repeated), the cannulas were removed, and the fermentation suspensions were incubated for 24 h in a shaking water bath at 37 °C. Afterward, the fermentation process was stopped by placing the suspensions on ice. Each sample was transferred to 50 mL tubes and centrifuged (4200*g*, 4 °C) for 30 min. The fermentation supernatants (F-AEs) were divided into aliquots and stored at −80 °C. Samples were sterilized by filtration (pore size 0.22 μm) before use in the cell culture experiments.

Analyses of Polyphenols or Metabolites in Fermented AE Samples. Only two (F-AE03 and F-AE04) of three fermentations samples were characterized analytically due to the limited sample size of F-AE02. Aliquots (2 mL) of the fermentation samples including the blank (without AEs) were lyophilized and dissolved in methanol. Polyphenol amounts in the fermentation samples were determined using the Hewlett-Packard 1100 HPLC gradient pump and a Hewlett-Packard 1100 photodiode array detector (Waldbronn, Germany), equipped with a Wisp 712b autosampler (Waters, Eschborn, Germany). Data acquisition and evaluation were performed with Hewlett-Packard ChemStation software. A Hypersil Gold C18 column, 100 mm × 4.6 mm, with a 3 μm particle size (Thermo, Runcorn, UK), was used. The mobile phase consisted of aqueous 0.1% v/v formic acid and ACN (Lichrosolv, Merck, Darmstadt, Germany). The gradient applied was 1−99% ACN in 40 min at a flow rate of 1 mL/min, and 25 μL injection volumes were used. The peaks were identified by comparison of retention time and UV spectra (200−600 nm) with authentic references (*22*). Chlorogenic acid, caffeic acid, 4-*p*-coumaroylquinic acid, phloretin-2′-*O*-xyloglucoside, phloridzin, phloretin, procyanidin B_1, procyanidin B_2, (+)-catechin, (−)-epicatechin, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinoside, quercetin-3-*O*-rhamnoside, quercetin, and quercetin-3-*O*-rutinoside (100 mg/L each) in methanol were diluted. Dihydrochalcones, catechins, and procyanidins (B_1 and B_2) were determined at 280 nm, hydroxycinnamic acid derivatives at 320 nm, and flavonols at 360 nm. 3,4,5-Trimethoxycinnamic acid (Sigma, Steinheim, Germany) was used as a standard (IS) for quantification (50 mg/L). Calibration curves (at the appropriate wavelengths according to the absorption maximum of the compounds) were used for quantification. Compounds were quantified by means of calibration curves (peak area divided by IS area vs quotient of substance and IS concentration). Linearity was given for 0.4−600 mg/L; limits of quantification ranged from 0.4 to 0.9 mg/L and limits of determination from 0.2 to 0.4 mg/L with a signal-to-noise ratio of 3:1, respectively.

Analysis of SCFA. To determine the SCFA, the samples were mixed with an isocapronate standard (1:11 v/v), shaken, and centrifuged at 6400*g* for 10 min at 4 °C. Then, the gas chromatographic measurements (GC 17A, Shimadzu, Duisburg, Germany) were performed using a 15 m FFAP column (Phenomenex, Aschaffenburg, Germany) and a specific temperature program (starting temperature 130 °C, increase 35 °C/min, and final temperature 170 °C) (*30*).

Human Colon Cell Lines LT97 and HT29. LT97 cells were isolated from a micro-adenoma of a patient with familiar adenoma polyposis coli (*26*). These are cells of an early colon adenoma in the

premalignant stage of tumor development. Concerning their genetic specifications, they are characterized by homozygous mutations of the *APC* tumor suppressor gene and a homozygous *k-ras* oncogene. However, there are no genetic changes in the *p53* gene. LT97 cells were cultivated in MCDB medium with 2% fetal calf serum (FCS), 10 μg/mL insulin, 2×10^{-10} M triiodotyronin, 2 mg/mL transferrin, 1 μg/mL hydrocortisone, 5×10^{-9} M sodium-selenite, 30 ng/mL epidermal growth factor (EGF), and 1% penicillin/streptomycin. Adherent cultures were passaged at subconfluent stages by using PBS/EDTA (5 mM).

The HT29 cell line that had been established by Fogh and Trempe from a colon adenocarcinoma of a Caucasian female (*31*) was originally purchased from the American Tissue Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified eagle medium (DMEM, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% FCS and antibiotics (1% penicillin/streptomycin (v/v); Roche Molecular Biochemicals, Meylan, France) according to our laboratory standard culture conditions. Confluent cultures were passaged 3 or 4 days after trypsinization.

Both cells lines were maintained under sterile conditions at 37 °C in a 95% humidified incubator (5% CO_2). Cells were routinely checked for mycoplasma contaminations using highly sensitive PCR analysis (Minerva Biolabs GmbH, Germany). For the experiments performed here, cells of passages 32−36 (LT97), and passages 30−41 (HT29) were used.

Determination of Cell Growth. Growth and survival of colon cells were determined in 96 well microtiter plates (Nunc GmbH & Co. KG, Berlin, Germany). A total of 72 h (LT97, ∼20−30% confluence) or 48 h (HT29 cells 8000 cell/well, ∼20−30% confluence) after seeding the cells, the cultures were treated with AEs (AE02, AE03, and AE04) and fermented AEs (F-AE02, F-AE03, and F-AE04) diluted in cell culture medium containing 0−850 μg/mL (dry mass) and 0−900 μg/mL (dry mass), respectively. After 24, 48, and 72 h of incubation time, the cells were lysed and fixed by methanol. Total cell counts were determined indirectly by staining DNA with DAPI (4′,6-diamino-2-phenylindole, Sigma, Germany), which becomes fluorescent after DNA binding. After 30 min, the DNA content, which reflects the number of cells, was detected by fluorimetrical analysis with Ex/Em 360/450 (TECAN Spectrafluor GmbH, Crailsheim, Germany). All data points were performed in triplicate, and each experiment was repeated independently at least 3 times for statistical evaluation. There were no additional substances added to the extracts. To measure the effect of AEs on cell growth, two controls were included in the proliferation assay. One was the no treatment control, for which the cells were incubated with only medium (0 μg/mL, 100%), and the second control was blank control, performed using different concentrations of extracts without cells. This second control (blank) was included to accommodate for the fluorescence of the extracts. Moreover, since DAPI can also produce fluorescence in the presence of extracts alone, the artifacts were normalized to the original fluorescence from cell DNA. The effective median doses (EC_{50}) of AEs and F-AEs that inhibited growth by 50% were determined and expressed as micrograms (μg) of AE.

Statistical Analysis. Statistical analysis was performed using the GraphPad Prism Version 4.0 for Windows (GraphPad Software Inc., San Diego, CA). Data shown in the tables and figures represent mean values ± SEM. Unless otherwise stated, these means were calculated from the means of triplicate replicates obtained in at least three independent experiments. Significant differences between treatment and control values were determined by one-way ANOVA and Bonferroni's post test.

RESULTS

Contents of Polyphenols in AE02, AE03, and AE04. The content of AE02 was reported in our previous publication (*10*), and it was used here to compare the content of new AEs (AE03 and AE04) additionally investigated here. AE02 contained the highest concentration of polyphenols, with a total amount of 533.9 mg/g of AE. AE04 contained 478.3 mg/g, whereas AE03 contained only 310.9 mg/g. **Table 1** shows the individual constituents in each of the three AEs. Chlorogenic acid was the most abundant phenolic compound detected in AE02 and AE04, each with concentrations of about 180 mg/g, whereas AE03 contained only about one-tenth of this amount. The phloretin sugars formed another abundant group of phenolic compounds in the AEs. AE02 contained the highest amount of phloretin glycosides with a total content of 200.5 mg/g. Phloridzin (phloretin-2′-*O*-β-D-glucoside) was the most common glycoside with 78.9 mg/g detected in AE03. AE02 had the smallest amount of phloridzin with only 27.9 mg/g. Quercetin derivates were detected in all three AEs. The total amounts of quercetin derivatives amounted to 116.2, 12.1, and 8.8 mg for AE03, AE04, and AE02, respectively. There are some uncertainties encountered for estimating the presence of unknown dihydrochalcones (see **Table 1** footnote), but according to the relevant literature, **Table 1** is more or less complete and contains all the major polyphenols. In addition, the concentrations of total sugar were also analyzed and are included in **Table 1**. Moreover, the extracts were analyzed for lipids after methanol/chloroform extraction. But, after a derivatization to fatty acid methyl ester (FAMEs) followed by gas chromatography−mass spectrometry (GC−MS) on a DB-Wax column, no fatty acids could be detected. Also, no phytosterols (silylation, GC−FID on DB-5) were detectable. Extracts were also analyzed for proteins, but after a 6 M HCl hydrolysis, no amino acids were detected in the hydrolysates (HPAEC/PAD, Dionex BioLC).

Table 1. Polyphenols in AE from Cultivar Years 2002, 2003, and 2004 (AE02, AE03, and AE04) Analytically Characterized by HPLC[a]

Substances	mean values (mg/g of AE)		
	AE02	AE03	AE04
280 nm			
procyanidin B1	7.0	6.2	n.d.
procyanidin B2	15.1	18.4	12.1
(+)-catechin	n.d.	2.7	n.d.
(−)-epicatechin	19.2	17.7	12.5
phloretin glycoside 1[b]	24.7	n.d.	n.d.
phloretin glycoside 2[b]	9.0	n.d.	n.d.
phloretin-2-*O*-xyloglucoside[c]	138.9	31.7	68.9
phloretin-2-*O*-xylogalactoside[c]		n.d.	4.2
phloridzin	27.9	78.9	48.0
320 nm			
chlorogenic acid	181.5	19.2	183.2
caffeic acid	4.8	4.0	7.5
3-coumaroyl-quinic acid[d]	9.5	3.0	9.4
4-coumaroyl-quinic acid[d]	77.3	5.0	66.0
5-coumaroyl-quinic acid[d]	10.4	3.8	39.8
p-cumaric acid	n.d.	4.2	2.6
cumaroyl-glucose	n.d.	n.d.	11.9
360 nm			
quercetine-3-*O*-rutinoside	2.6	49.1	4.5
quercetine-3-*O*-galactoside	0.8	8.1	1.8
quercetine-3-*O*-glucoside	1.4	12.3	1.5
quercetine-3-*O*-rhamnoside	4.1	25.1	4.3
quercetine-3-*O*-xyloside	n.d.	18.1	n.d.
quercetine-3-*O*-arabinopyranoside	n.d.	3.5	n.d.
total polyphenols	533.9	311.0	478.2
total polysaccharides	164.0	305.0	99.0

[a] Content of AE02 was reported in our previous publication (*10*). We have included the data here to compare the content of AE02 to the additionally investigated AEs (AE03 and AE04). n.d.: Polyphenols that were not detectable. [b] No commercial standard available, glycoside part unknown. [c] No commercial standard available, isolated by preparative HPLC, unknown dihydrochalcone. [d] No commercial standard available, quantitated with 3-isomer.

Contents of Polyphenols or Metabolites and SCFA in the Fermentation Samples. The polyphenols were hardly detectable

Table 2. Polyphenols and Metabolites Present in Fermented AE (F-AE03 and F-AE04) Analytically Characterized by HPLC[a]

	before fermentation		after fermentation	
polyphenols and metabolites (mg/g)	AE03	AE04	F-AE03	F-AE04
procyanidin B2	18.4	12.1	0.024	n.d.
catechin	2.7	n.d.	0.042	n.d.
phloretin-2-xyloglucoside	31.7	68.9	0.035	n.d.
phloridzin	78.9	48.0	n.d.	0.001
caffeic acid	4.0	7.5	n.d.	0.001
quercetin-3-rhamnoside	25.1	4.3	0.003	n.d.
phloroglucin	n.d.	n.d.	42.2	n.d.
3,4-dihydroxyphenylpropionic acid	n.d.	n.d.	33.3	41.3
total	160.80	140.80	75.60	41.30

[a] There were no polyphenols detected in the fermented blank control sample (without addition of AEs). n.d.: Polyphenols or metabolites were not detected.

in the fermentation samples (**Table 2**). Most of the polyphenols were degraded (approx. 99.9%) when compared to the total amount of non-fermented AEs. The total amounts were 0.10 and 0.002 mg/L of fermented F-AE03 and F-AE04, respectively. Only some of the polyphenols that have complex structures, namely, (+)-catechin (0.145 μmol/L), phloretin-2′-*O*-xyloglucosid (0.062 μmol/L), procyanidin B_2 (0.041 μmol/L), quercetin-3-*O*-rhamnoside (0.007 μmol/L), phloridzin (0.002 μmol/L), and caffeic acid (0.006 μmol/L) were retrieved in very minor amounts in the sample F-AE03 and F-AE04, respectively. There were no polyphenols detected in the fermented blank control samples (without addition of AEs). Moreover, the analysis of polyphenol metabolites in the fermentation samples showed that the total concentration of phloroglucin and 3,4-dihydroxyphenylpropionic acid was higher in F-AS03 (75.5 mg/L) than in F-AS04 (41.3 mg/L).

The formation of SCFA in the control sample (prepared without the addition of AE) was lower (11.4 mmol/L) than in the fermented samples with AE (**Figure 1**). The mean content of total SCFA in F-AE02 (44.5 mmol/L) was approximately 1.5-fold higher than in the other samples, which amounted to 28.9 mmol/L in F-AE03 and 35.4 mmol/L in F-AE04. Among all the analyzed SCFA, acetate (29.16 mmol/L) was found in high concentrations in all three F-AEs in comparison to the fecal control (blank), which had been produced without addition of the AEs. Butyrate (5.7 mmol/L) and propionate (4.5 mmol/L) were the second most abundant SCFA in all the F-AEs. The relative molar ratios for acetate, butyrate, and propionate were 65:19:16 for the blank, 74:15:11 for AE02, 69:15:16 for AE03, and 76:14:10 for AE04.

Modification of LT97 and HT29 Cell Growth by AEs. The effect of AE02 on HT29 cells was reported in our previous publication (*10*), and here, its activities were compared to AE03 and AE04. Treatment of LT97 and HT29 cells with all three AEs affected the cell growth in a time- and dose-dependent manner (**Figure 2**). After 24 and 48 h of treatment, the growth of the LT97 adenoma cells was more strongly inhibited by AEs than in the HT29 carcinoma cells. After 48 h, the EC_{50} values ranged from 240.8 ± 28.0 −to 454.8 ± 59.9 μg and from 380.8 ± 18.5 −to 634.5 ± 42.8 μg for the different AEs in the LT97 and HT29 cells, respectively (**Table 3**). AE03 had the highest antiproliferative activity as compared to AE02 or to AE04 and resulted in EC_{50} values of 240.8 ± 28.0 and 380.8 ± 18.5 μg after 48 h, in LT97 and HT29 cells, respectively. AE02 had the lowest antiproliferative activities with EC_{50} values of 454.8 ± 59.9 and 634.5 ± 42.8 μg after 48 h in LT97 and HT29 cells, respectively. Intermediate bioactivities were observed for AE04 (**Table 3**) with EC_{50} values of 290.6 ± 18.7 or 411.9 ± 30.8 μg in LT97 or HT29 cells. The values, however, were not significantly different from the values obtained for AE02 and AE03.

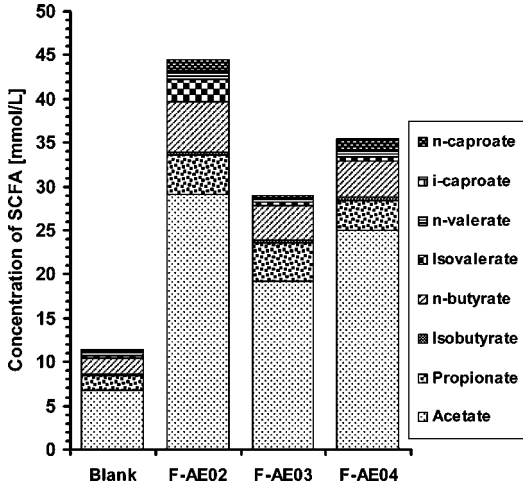

Figure 1. SCFA (mmol/L) in the fermentation samples of AEs (F-AE02, F-AE03, and F-AE04), $n = 2$.

Modulation of LT97 and HT29 Cell Growth by Fermented AEs. Figure 3 shows the effects of F-AEs on the growth of LT97 and HT29 cells after 24 and 48 h of treatment. The effect on cell growth of F-AEs and of the corresponding fermentation blank (without addition of AEs) was measured. Since there was a significant ($p \leq 0.01$) inhibition of cell growth by the blank (after 48 h), the data of F-AEs were normalized to the corresponding F-AE blank. All fermented samples also inhibited the growth of LT97 and HT29 cells in a time- and concentration-dependent manner. However, again, F-AE03 showed a more pronounced inhibitory effect than F-AE02 and F-AE04 and resulted in EC_{50} values of 404.1 ± 140.8 and 801.0 ± 44.0 μg after 48 h, in LT97 and HT29 cells, respectively (**Table 4**). The results show that the growth inhibition by fermented AEs did not directly reflect the amounts of SCFA found in the samples (**Figure 1**). Although the mean value of SCFA for F-AE02 was higher than that of F-AE03 and F-AE04, this did not result in a stronger growth inhibition. The LT97 cells were more sensitive than the HT29 cells toward the growth inhibitory activities of all F-AE. As compared to unfermented AEs, the F-AEs were approximately 3-fold less active.

DISCUSSION

AEs contain several compounds with antiproliferative potential (*10*). In this study, we used three different types of AEs, each containing different concentrations and types of polyphenols. The analyses of polyphenols again provided evidence for remarkable differences depending on cultivars, varieties, and harvest years.

The polyphenols, however, seem to be mostly degraded by fermentation mediated through the gut flora since some compounds were no longer detectable in fermented AEs. Exceptions were for larger, more complex polyphenols such as catechin and procyanidin derivatives, which were both still detectable, although only in very small amounts. Because of the complex structures of these particular compounds, they might be less susceptible to the action of the gut microflora enzymes (*32*). In addition, the fermentation process resulted in the formation of SCFA. Thus, by the fermentation of apple polyphenols through the gut flora, SCFA can be produced in the human colon, and

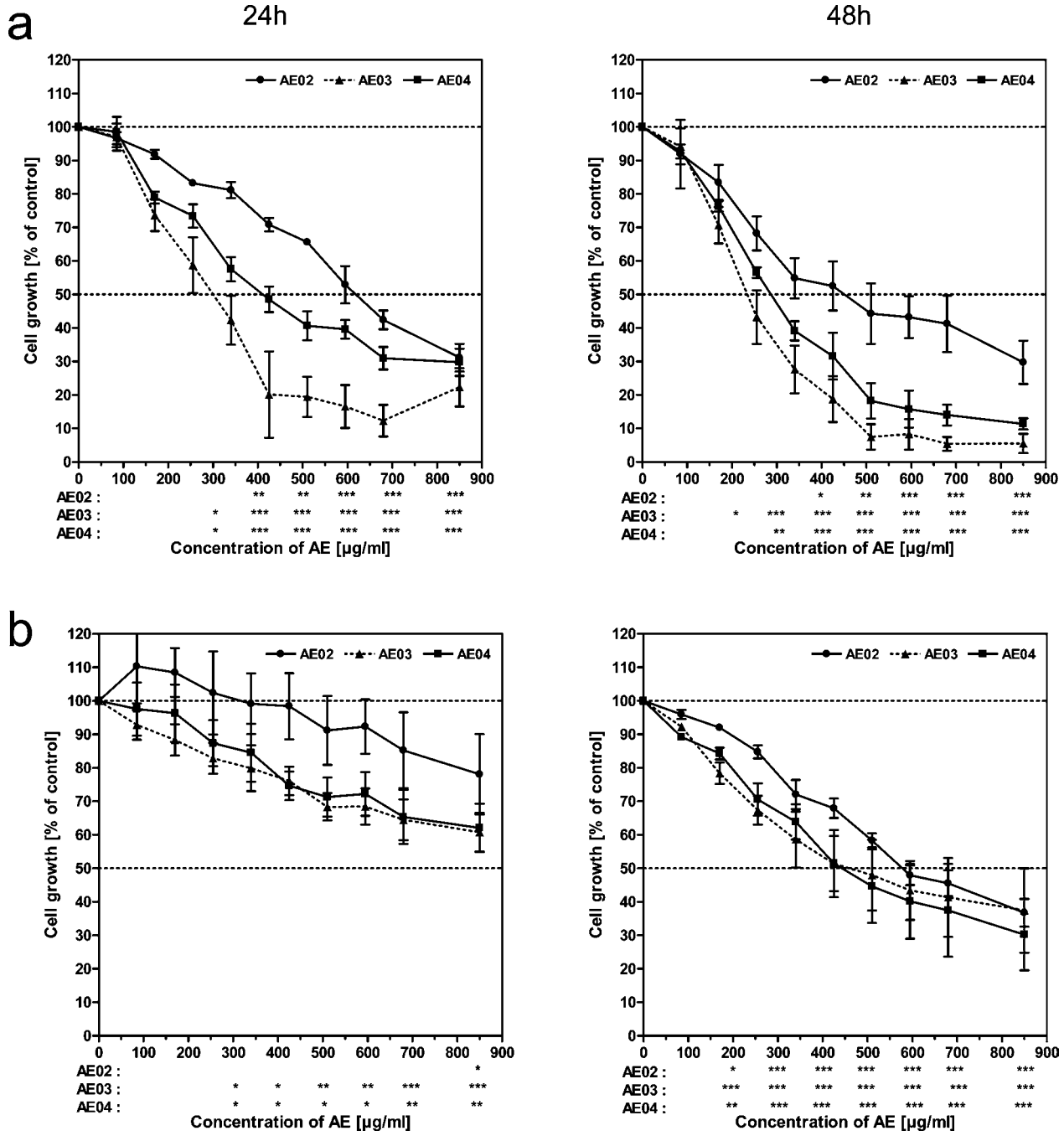

Figure 2. Growth inhibition of LT97 colon adenoma cells (**a**) and HT29 colon carcinoma cells (**b**) after 24 and 48 h incubation with AEs. The effect of AEs was normalized to the blank control (AEs without cells) and to the non-treated control (0 μg/mL, 100%) cells. One-way ANOVA and Bonferroni's post test gave *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$, $n = 3$.

Table 3. Antiproliferative Activities (EC_{50} Values, μg) of AEs Determined in LT97 and HT29 Cells[a]

	EC_{50} (μg/mL)					
AE	LT97			HT29		
treatment time (h)	24	48	72	24	48	72
AE02	650.9 ± 25.2	454.8 ± 59.9	361.0 ± 59.3	×	634.5 ± 42.8	462.4 ± 20.2
AE03	280.6 ± 45.3	240.8 ± 28.0	206.2 ± 19.5	×	380.8 ± 18.5	272.5 ± 8.1
AE04	430.8 ± 26.6	290.6 ± 18.7	224.2 ± 15.3	×	411.9 ± 30.8	454.7 ± 181.3

[a] ×: Denotes that EC_{50} values were not achieved, $n = 3$.

the formation depends on the amount of total polyphenols present in AEs. F-AE02, which had the highest amount of polyphenols before fermentation, also contained the highest amount of total SCFA. Interestingly, the fermentation of dietary fibers such as prebiotic long chain inulin-type fructans or arabinoxylans resulted in quite similar SCFA profiles (*33*, *34*).

We used DNA-DAPI staining as an indirect method to assess the total cell number in the proliferation assay. We have chosen

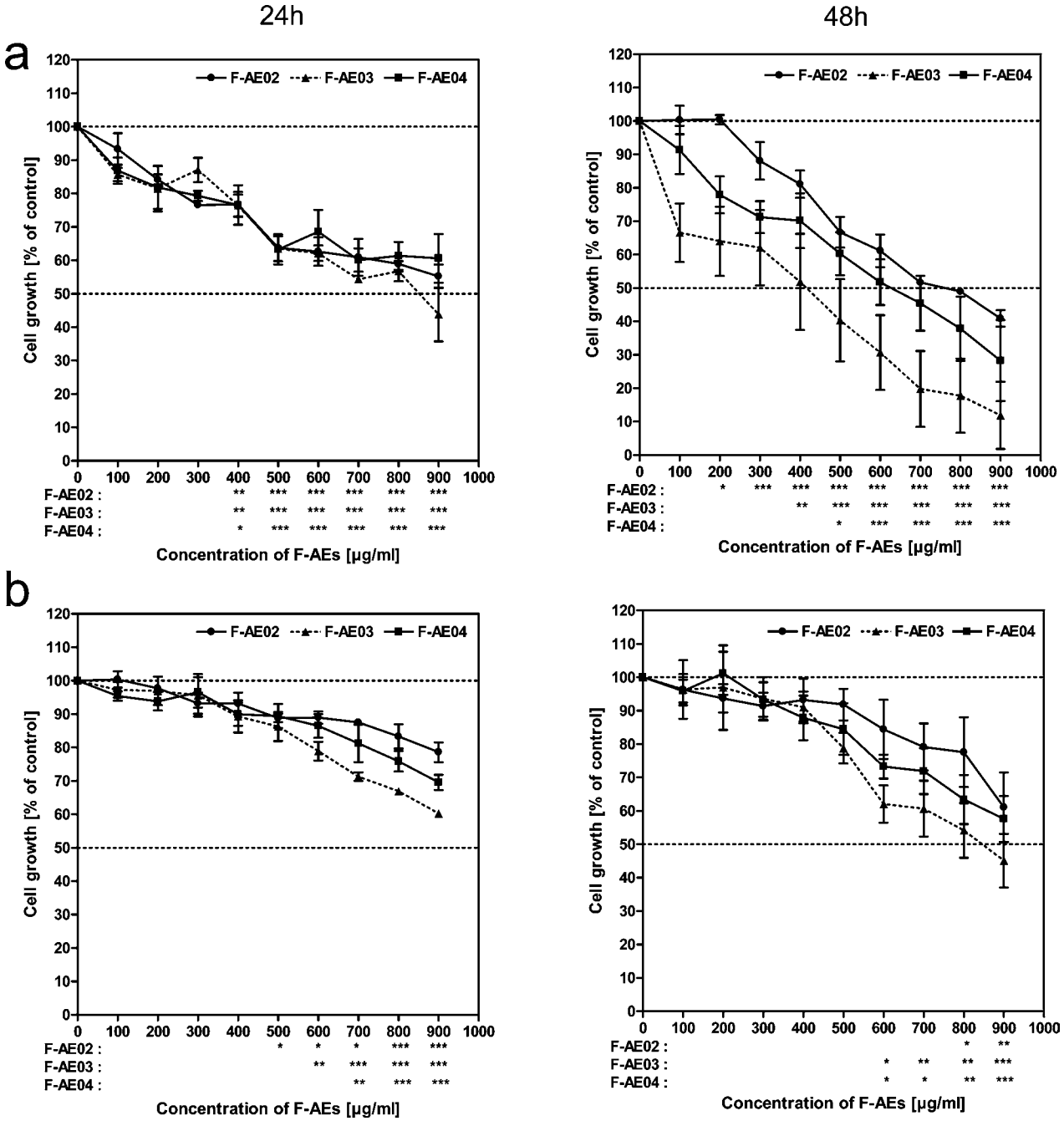

Figure 3. Growth inhibition of LT97 colon adenoma cells (**a**) and HT29 colon carcinoma cells (**b**) after 24 and 48 h incubation with F-AEs. The effect of AEs was normalized to the blank control (AEs without cells) and to the non-treated control (0 μg/mL, 100%) cells. One-way ANOVA and Bonferroni's post test gave $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$, $n = 3$.

Table 4. Antiproliferative Activities (EC_{50} Values, μg) of Fermented AEs Determined in LT97 and HT29 Cells[a]

	EC_{50} (μg/mL)					
F-AE	LT97			HT 29		
treatment time (h)	24	48	72	24	48	72
F-AE02	×	764.7 ± 9.3	628.8 ± 40.3	×	×	738.2 ± 13.2
F-AE03	821.4 ± 49.5	404.1 ± 140.8	565.8 ± 99.0	×	801.0 ± 44.0	687.7 ± 18.0
F-AE04	784.9 ± 44.1	572.4 ± 52.8	398.3 ± 106.4	×	×	724.9 ± 82.6

[a] ×: Denotes that EC_{50} values were not achieved, $n = 3$.

this method since it is faster, more reliable, and sensitive to determine the total cell number as compared to other methods used routinely in the laboratory. To exclude possible artifacts arising from fluorescence of the test substance, we included a blank control (test substances without cells). In our study, we used two in vitro cell model systems. One is a highly transformed human colon carcinoma cell line (HT29), and the second is a human colon adenoma cell line (LT97) that is

representative of preneoplastic cells. The rationale of the model choice was to compare the antiproliferative efficacy of apple polyphenols between transformed cells and partially transformed cells. If the effect was more pronounced in preneoplastic cells than highly transformed cells, one could expect that the effect would be higher in primary cells. However, since both cell types were grown and maintained in different cell culture media, some of the differences could also be attributed to culture conditions. The antiproliferative activity of AE03 was higher than that of AE04 and AE02 in both LT97 and HT29 cells after 24 and 48 h treatments. This was unexpected since AE03 (311.0 mg/g) contained only approximately 65 or 58% of the total polyphenols contained in AE04 or AE02, respectively. An explanation for this finding, however, could be that the pronounced antiproliferative activities of AE03 result from its higher quercetin concentrations, which were about 10- and 13-fold higher than the respective concentration of AE04 and AE02. This is supported by findings from previous studies with HT29 cells, showing that quercetin was the major compound contributing to the antiproliferative and antioxidative activities of AEs (*10*, *35*, *36*). This present part of the study confirms previous findings that both the mixture of major apple flavonoids as well as the amount of specific bioactive flavonoids are important factors for growth arrest in human colon cell lines (*37*).

Since the majority of the polyphenols was degraded (99.9%) during human gut flora-mediated fermentation, the growth inhibitory effects of F-AEs in LT97 and HT29 cells are probably not due to the same polyphenols as they are in the unfermented samples. The amounts of the detected polyphenols, such as (+)-catechin (0.145 μmol/L), phloretin-2′-*O*-xyloglucoside (0.062 μmol/L), procyanidin B_2 (0.041 μmol/L), and quercetin-3-*O*-rhamnoside (0.007 μmol/L), are possibly below the effective concentration ranges found in previous investigations, and some are lower than the reported concentration of apple polyphenols in human plasma (0.1−0.4 μmol/L) (*38*−*40*). It will, however, be of interest to assess in the future whether such low amounts of polyphenols, together with resulting metabolites, are able to inhibit growth or modulate the expression of relevant genes, as has, for example, been reported before (*41*, *42*). For example, it has also been shown that exposure of colon cells to catechins and procyanidins increases the expression of phase II enzymes, such as GSTs and UGTs, that are important for the biotransformation of carcinogens (*43*, *44*).

The SCFA, and especially butyrate, produced from AEs may play a role in the colon. Colon crypts may use SCFA as an energy source, whereas in tumor cells, SCFA stimulate pathways of growth arrest, differentiation, and apoptosis (*45*). Moreover, SCFA may also enhance toxicological defense in primary, adenoma, and tumor human colon cells by favorably modulating detoxifying enzymes (*46*). In our investigation, we did not find an association between the concentration of SCFA and antiproliferative properties of AEs. The F-AE03 contained only 80 and 63% of the SCFA contained in F-AE04 and F-AE02, respectively, but F-AE03 inhibited cell growth 1.6- and 1.4-fold more efficiently than F-AE04 and F-AE02 in LT97 cells. Similar directional but less pronounced effects were noticed with F-AE03 in comparison to F-AE04 and F-AE02 in HT29 cells with 1.2- and 1.1-fold differences, respectively. Thus, the amount of SCFA present in F-AEs did not directly reflect the differences in antiproliferative activities of F-AEs. Moreover, the total concentration of 3,4-dihydroxyphenylpropionic acid and phloroglucin, which are metabolites of proanthocyanidins and phloridzin (*47*, *48*) polyphenols, is higher in F-AS03 (75.5 mg/L) than in F-AS04 (41.3 mg/L), respectively. This might cause differences in antiproliferative effects by fermented AEs.

On the basis of equivalent apple concentrations, AEs were consistently about 10-fold more growth inhibitory than F-AEs in both LT97 and HT29 cells. Thus, the fermentation process reduced the effectiveness of AEs. The growth inhibition of adenoma-derived LT97 was more pronounced than carcinoma-derived HT29 cells after treatment with both AEs and F-AEs. LT97 cells may be representative of preneoplastic lesions in the human colon (*26*); thus, apple polyphenols might have a higher antiproliferative efficacy in the preneoplastic lesion than in carcinoma cells.

In conclusion, apple polyphenols were able to significantly suppress the growth of both adenoma (LT97) and carcinoma cells (HT29). The growth suppressing properties of AEs are due to their polyphenols but not due to the SCFA derived from the polyphenols during gut flora-mediated fermentation. The adenoma cells were more sensitive than the highly transformed carcinoma cells. This may mirror a higher chemoprotective potential of apple polyphenols in the preneoplastic lesion than in carcinoma. Our findings indicate that the adenoma and carcinoma cell proliferation is significantly inhibited by a specific combination of an apple polyphenol/flavonoid mixture from different cultivars. Collectively, these results imply that the antiproliferative effect by AEs might contribute to its overall chemoprotective function against colon carcinogenesis.

ABBREVIATIONS USED

AEs, apple polyphenol extracts; AE02, apple extract 2002; AE03, apple extract 2003; AE04, apple extract 2004; F-AEs, fermented apple polyphenol extracts; SCFA, short chain fatty acid.

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