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Antioxidant Effectiveness of Phenolic Apple Juice Extracts and Their Gut Fermentation Products in the Human Colon Carcinoma Cell Line Caco-2

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Apples represent a major dietary source of antioxidative polyphenols. Their metabolic conversion by the gut microflora might generate products that protect the intestine against oxidative damage. We studied the antioxidant effectiveness of supernatants of fermented apple juice extracts (F-AEs, 6 and 24 h fermentation) and of selected phenolic degradation products, identified by HPLC-DAD-ESI-MS. Cell free antioxidant capacity of unfermented apple juice extracts (AEs) was decreased after fermentation by 30−50%. In the human colon carcinoma cell line Caco-2, F-AEs (containing <0.5% of original AE-phenolics) decreased the reactive oxygen species (ROS) level more efficiently than the F-blank (fermented without AE) but were less effective than the respective AEs. Similarly, antioxidant effectiveness of individual degradation products was lower compared to respective AE constituents. Glutathione level was slightly increased and oxidative DNA damage slightly decreased by fermented AE03, rich in quercetin glycosides. In conclusion, F-AEs/degradation products exhibit antioxidant activity in colon cells but to a lesser extent than the respective unfermented AEs/constituents.

KEYWORDS: Anaerobic fermentation; apple polyphenols; Caco-2 cells; cellular ROS-level; colon; Comet assay; HPLC-DAD-ESI-MS; glutathione; TEAC

INTRODUCTION

The etiology of many diseases of the gastrointestinal tract, such as colon cancer or inflammatory bowel diseases (Crohn's disease, ulcerative colitis), is associated with an imbalance in the cellular redox system leading to an increased level of reactive oxygen species (ROS). For colorectal cancer, epidemiological studies have shown an inverse correlation between consumption of fruits and vegetables and disease risk, mainly attributed to high ingestion of secondary plant metabolites (e.g., polyphenols) (*1*). The preventive potential of polyphenols is, at least in part, due to their ability to scavenge reactive oxygen species (ROS) and thus to protect the organism against oxidative stress mediated damage (*2*). In Europe, dietary polyphenol intake is about 1 g per day, estimated to consist of two-thirds flavonoids and one-third phenolic acids (*3*). Apples (and apple juice) represent a major source of such polyphenols whose main classes are flavonoids such as quercetin glycosides (e.g., rutin, Rut), hydroxycinnamic acids, and esters (e.g., chlorogenic acid (ChA, 5-caffeoylquinic acid), procyanidins, and dihydrochalcones, e.g. phloridzin (Pz, phloretin-2′-β-D-glucoside)) (*4*). In apple juice, phenol contents range between 154 and 970 mg/L, dependent on the apple variety under study (*5*).

Food derived polyphenols are extensively degraded/metabolized by gut enzymes and microflora, as shown in vitro (*6, 7*) and in human studies. After consumption of apple and red grape juices, vegetables (e.g., onions, tomatoes), tea, and coffee, mainly phenolic acids were identified as metabolites in plasma, urine, and fecal water of healthy probands (*8–10*). A study with ileostomy patients consuming cloudy apple juice showed that most of the ingested polyphenols were absorbed or metabolized in the small intestine and that up to 33% of the parent compounds would reach the colon under physiological conditions (*11*). Dietary polyphenols are substantially metabolized by the intestinal microflora and by brush border- and intracellular enzymes such as hydrolases or transferases (*6, 7, 9*). Quercetin

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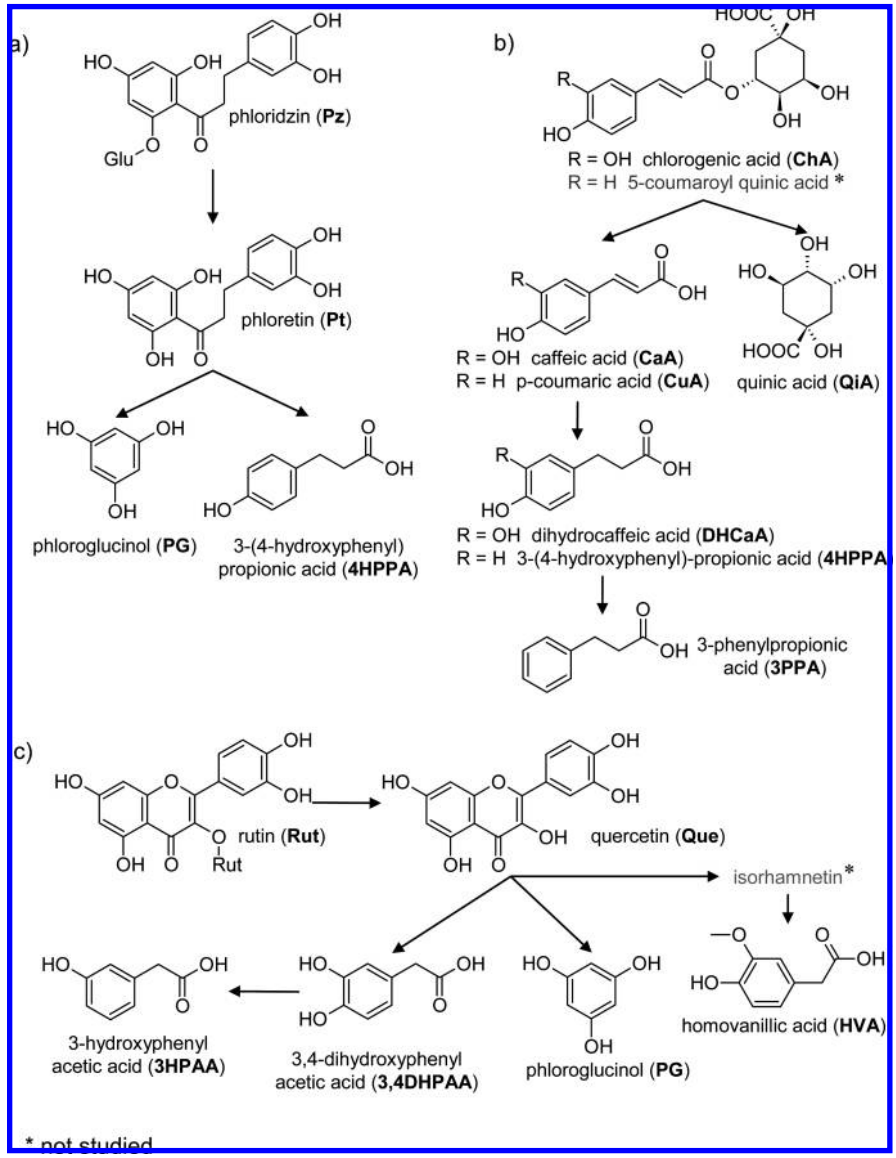
Scheme 1. Proposed Degradation/Metabolism of Selected Apple Phenolics in the Human Gut (*6–9, 12*)



glycosides are primarily deglycosylated to quercetin (Que), followed by several degradation steps including fission of the C-ring to generate compounds, such as 3,4-dihydroxyphenylacetic acid (3,4DHPAA), 3-hydroxyphenylacetic acid (3HPAA), phloroglucinol (PG), and homovanillic acid (HVA, 4-hydroxy-3-methoxyphenylacetic acid), as shown in **Scheme 1** (*6, 9, 12*). ChA is first cleaved at the ester bond to caffeic acid (CaA), followed by reduction of the aliphatic double bond forming dihydrocaffeic acid (DHCaA, 3-(3,4-dihydroxyphenyl)propionic acid) and by aromatic dehydroxylation to 3-phenylpropionic acid (3PPA) (*9, 12*). The dihydrochalcone glucoside Pz, another characteristic apple juice constituent, is first deglycosylated, and the resulting aglycon phloretin (Pt, 2′,4′,6′-trihydroxy-3-(4-hydroxyphenyl)propiophenone) is further degraded to products identified as PG and 3-(4-hydroxyphenyl)propionic acid (4HPPA) (*6, 7*). Several in vitro studies with colonic bacteria reveal that the large group of polyphenols is degraded to a limited number of products, predominantly phenolic acids (*6, 13*). However, knowledge on the biological effectiveness of these metabolites in the intestine is still limited (*14, 15*).

Cloudy apple juice was found to decrease DNA damage, formation of aberrant crypt foci (ACF), and hyperproliferation in the gut of rats treated with the colon carcinogen 1,2-dimethylhydrazine; these effects have been ascribed to polyphenolic and nonpolyphenolic constituents (*16*). In a study by Veeriah et al. (*17*), two different phenolic apple juice extracts (AEs), fermented for 24 h with human fecal flora, were found less active in reducing survival of human colon adenoma (LT97) and carcinoma (HT-29) cells than the corresponding unfermented extracts.

The present study addresses the question whether intestinal degradation modulates the antioxidant effectiveness of apple juice phenolics. To mimic the situation within the colon, AEs fermented with human fecal slurry (*17*) were tested in comparison to the respective unfermented AEs. The selected AEs substantially differed in their polyphenol pattern: AE04 has a high amount of hydroxycinnamic esters whereas the pomace extraction juice AE03 is especially rich in quercetin glycosides. This provides an insight into the differential colonic degradation of these important groups of apple polyphenols and its impact on antioxidant capacity. Feces, fermented without AEs, were included as controls to assess the influence of other fecal constituents. In the human colon adenocarcinoma cell line Caco-2, which shows biochemical characteristics of normal adult intestine and colon cells, the cellular markers ROS level (DCF assay), oxidative DNA damage (Comet assay), and glutathione level (photometric kinetic assay) were studied. Fermentation supernatants of AEs (F-AEs), resulting from 6 h fermentation,

were included to evaluate the influence of fermentation time on biological activity. Cell free antioxidant capacity (trolox equivalent antioxidant capacity, TEAC) was also determined to distinguish between direct radical scavenging activity and influence of cellular response mechanisms. Known intestinal degradation products (mainly phenolic acids) and respective parent compounds (Que glycosides, ChA, Pz) were similarly examined for modulation of cellular ROS level (DCF assay) and of TEAC. Some incubations were also performed in the presence of catalase to elucidate whether artifactual hydrogen peroxide formation, resulting from pro-oxidative interaction of phenolic test compounds with cell medium constituents (e.g., transition metal ions), affects the cellular ROS level (*18, 19*). Additionally, F-AEs were submitted to HPLC-DAD-ESI-MS (high-performance liquid chromatography-diode array detection-electrospray ionization-mass spectrometry) analysis to quantify the amount of residual AE constituents and of metabolites/degradation products.

MATERIALS AND METHODS

Chemicals, Cells, and Media. Menadione (Md), *tert*-butylhydroperoxide (TBH), reduced/oxidized glutathione, glutathione reductase (GSR), catalase, ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), potassium persulfate, DTNB (5,5′-dithiobis(2-nitrobenzoic acid)), 2-vinylpyridine, 5-sulfosalicylic acid (SSA), ethidium bromide, triethanolamine (TEA), Rut, Que, 3,4DHPAA, 3HPAA, HVA, ChA, CaA, 3PPA, 3,4-dihydroxybenzoic acid, 4-methylcatechol, (−)-epicatechin, isoferulic acid, Pz, and *p*-coumaric acid (CuA) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), NADPH, DMSO, 4HPPA, DHCaA, PG, D-(−)-quinic acid (QiA), ferulic acid, phenylacetic acid, Que-3-glucoside, Que-3-rhamnoside, and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were obtained from Fluka (Deisenhofen, Germany). Low and normal melting agarose were acquired from Bio-Rad (Munich, Germany), Pt, quercetin-3-galactoside, and (+)-catechin from Carl Roth GmbH (Karlsruhe, Germany), 3-(3-hydroxyphenyl)propionic acid (3HPPA) from Lancaster (Karlsruhe, Germany), and the BCA protein quantification kit from Uptima (Montluçon, France). Quercetin-3-xyloside, quercetin-3-arabinoside, and phloretin 2′-*O*-xyloglucoside as well as procyanidins B1 and B2 were kindly provided by Prof. Dr. H. Becker (Saarbruecken, Germany) and Prof. Dr. P. Winterhalter (Braunschweig, Germany). All solvents and chemicals were of analytical grade or complied with the standards needed for cell culture experiments. Formamidopyrimidine-DNA-glycosylase (FPG) was provided by A. R. Collins (Oslo, Norway). Caco-2 cells were obtained from Deutsche Sammlung fuer Mikroorganismen and Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and DMEM/F-12 (1:1) medium, fetal calf serum (FCS), and penicillin/streptomycin from Invitrogen (Karlsruhe, Germany). Cell culture consumable material (cell culture flasks, Petri dishes, well plates, etc.) were purchased from Greiner Bio-One (Essen, Germany).

Preparation of the Phenolic Apple Juice Extracts and Anaerobic Fermentation. Phenolic apple juice extracts AE03 (pomace extraction juice from table apple varieties, harvested 2003) and AE04 (mixture of different juices and apple varieties, harvested 2002 and 2003) were produced from juices of different apple varieties, harvested at Geisenheim Research Center and from local orchards as described (*20, 21*). Briefly, after crushing and extraction, the juices were separated and filtered. After adsorption of the polyphenols on adsorber resins and elimination of sugars, organic acids, and minerals with water, the polyphenol fraction was eluted with ethanol, concentrated, freeze-dried, and stored cool, excluding light and moisture.

Fermentation of AE03 and AE04 (10 g/L fecal suspension) was conducted in vitro under anaerobic conditions, according to described procedures by use of fresh human feces pooled from three healthy volunteers (*17*). A negative control (F-blank, fermented without AE) was included in each fermentation process. Fermentation was performed for 6 and 24 h, generating aqueous fermentation supernatants (F-AEs (6 and 24 h)), which were sterilized by filtration (pore size 0.22 μm) and stored at −80 °C until use.

Analysis of AE Constituents and Degradation Products in F-AEs. *1. AE Constituents.* Lyophilized fermentation samples were redissolved in methanol and analyzed for phenolic AE constituents using a HPLC-Diode Array System (Hewlett-Packard 1100) equipped with a Hypersil Gold C18 column (100 mm × 4.6 mm, 3 μm particle size) as described (*5, 17*). 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.

2. Degradation Products. Metabolites/degradation products were separated, according to Schieber et al. (*22*) with slight modifications using a Jasco HPLC-DAD system (PU-980, MD-2015 Plus; Jasco, Gross-Umstadt, Germany) with an Aqua C-18 column (250 mm × 4.6 mm, 5 μm particle size), equipped with a 4 mm × 3 mm C-18 ODS security guard (Phenomenex, Aschaffenburg, Germany). Eluents consisted of the following: A, 0.1% formic acid in water (v/v); B, methanol with the gradient 0 min 5% B, 5 min 20% B, 20 min 35% B, 40 min 58.5% B, 43 min 99% B at a flow rate of 1.2 mL/min. Linearity was given for 0.2 to 1280 mg/L; limits of quantification ranged from 0.3 to 2.5 mg/L and limits of determination from 0.2 to 1.25 mg/L.

Phenolic AE constituents and degradation products (1. and 2.) were identified by HPLC-DAD-ESI-MS comparing retention time, UV-(200−600 nm) and mass spectra with authentic references. Wavelengths used for DAD-quantification were 370 nm for flavonols (Que and glycosides), 320 nm for hydroxycinnamic acids, 280 nm for dihydrochalcones (Pz, Pt), phenolic acids (hydroxyphenylbenzoic, -acetic and -propionic acids), catechins ((−)-epicatechin, (+)-catechin), and procyanidins (B1 and B2), and 210 nm for PG and 3PPA. Compounds were quantified by means of calibration curves and using 3,4,5-trimethoxycinnamic acid (320 nm) as internal standard (IS) (peak area divided by IS area vs quotient of substance and IS concentration).

3. HPLC-ESI-MS Analysis. HPLC-ESI-MS analysis was performed with a SCIEX API 3200 LC/MS/MS tandem mass spectrometer equipped with an electrospray (ESI) interface (Applied Biosystems, Darmstadt, Germany) and a Jasco pump PU-980. Data acquisition and evaluation were conducted using Analyst Software 1.4.2 (Applied Biosystems, Darmstadt, Germany). HPLC chromatographic separations were carried out as described above (2.). The analysis was performed under following conditions: negative ionization mode; spray capillary voltage, 4.5 kV; curtain gas, nitrogen, 450 °C, 25 psi; GS2, 40 psi; electron multiplier voltage, 2.2 kV; declustering potential, −40 V; entrance potential, −4.5 V; GS1, nitrogen, 50 psi. The mass spectrometer was operated in full scan mode, 120−900 u, with total scan duration of 1.0 s and a dwell time of 2 ms. The obtained molecular ion peaks were compared to those of the standards.

Trolox Equivalent Antioxidant Capacity (TEAC). TEAC of AEs, their fermentation supernatants, and degradation products was determined by ABTS radical decolorization assay, as described previously (*21*).

Briefly, ABTS radical solution was prepared by activation of ABTS with potassium persulfate and adjusted to an extinction of 0.7 ± 0.02 with PBS. AEs and degradation products, dissolved in DMSO, were added to the ABTS radical solution, giving final concentrations of 0−15 μg/mL and 0−15 μM, respectively. F-AEs, diluted in PBS, were used in concentrations of 0−15%. After 6 min at 30 °C, absorbance was measured at 734 nm. Solvent controls and Trolox (antioxidant standard) curve were run in each assay. TEAC values are expressed in millimolar Trolox, equivalent to the antioxidant capacity of a solution containing 1 mg/mL AEs, 1 mM solution of degradation products, or 10% F-AEs (equivalent to a concentration of 1 mg/mL AE).

Cell Culture. Caco-2 cells were maintained in 175 cm^2 flasks in DMEM/F12 (1:1) supplemented with 20% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37 °C, 5% CO_2, and 95% relative humidity.

Incubation with (Fermented) AEs and Degradation Products. Caco-2 cells were seeded in Petri dishes, (glutathione determination: 10^6 cells per 9.6 cm dish; Comet assay: 2.5×10^5 cells per 6 cm dish), cultivated for 24 h, washed with PBS, and incubated with AEs (dissolved in DMSO; final concentration: 0.1%) or fermentation supernatants for 24 h in incubation medium containing 10% FCS. After incubation, cells were washed with PBS and isolated by trypsin (0.5% w/v) treatment. Cell suspensions were directly used for analysis of

glutathione level; for determination of oxidative DNA damage, cells were treated with the redox-cycler menadione prior to isolation, as described below. For determination of cellular redox status (DCF assay), cells were seeded in 48-well plates (2.4×10^4 cells per well) and incubated with AEs (dissolved in DMSO; final concentration: 1%), degradation products (both dissolved in DMSO), or F-AEs, as described above.

Some incubations were also performed in the presence of catalase (100 U/mL) added to the incubation medium.

Cellular ROS Level (DCF-Assay). Oxidative stress in cells was quantified by the DCF assay according to Wang and Joseph (*23*). Briefly, cells were washed with PBS after incubation, treated for 30 min with DCFH-DA (final concentration 50 μM in PBS pH 7.0; 1% DMSO v/v), washed and treated with TBH (250 μM in PBS) for 30 min at 37 °C. The increase of fluorescence (FI) was measured at 0 and 30 min after TBH addition in a microplate reader. All treatments and fluorimetric determination were performed in the dark. FI was calculated as described (*23*) and expressed as relative FI in percent of TBH-treated control.

GSH Level (Photometric Kinetic Assay). Total glutathione (tGSH = GSH + GSSG) and GSSG were measured by photometric determination of 5-thio-2-nitrobenzoate (TNB), formed from DTNB by GSH, as described previously (*21*). Briefly, cell suspensions were washed and resuspended in 370 μL of phosphate buffer. Aliquots (2×10 μL) were used for protein quantification. The remaining 350 μL was used for protein precipitation (350 μL of 10% SSA), centrifugation (12 000*g*, 4 °C, 15 min) and photometric determination of tGSH and GSSG. A freshly prepared reaction mixture (190 μL), containing 0.63 μM DTNB, 0.42 μM NADPH, and 0.53 U/L GSR in phosphate buffer, was added to 10 μL of centrifugation supernatant. GSH-dependent TNB formation was monitored in a microplate reader at 412 nm after 2 min reaction time. For GSSG analysis, 250 μL of the supernatants was treated with 50 μL of 50% TEA and 10 μL of 2-vinylpyridine for 1 h in a thermomixer at 26 °C, prior to photometric determination (20 μL aliquots, 10 min reaction time). tGSH was calculated as nanomoles per milligram of protein and expressed as percent of untreated solvent-control. Glutathione status was calculated as reduced GSH in percent of tGSH.

Oxidative DNA Damage (Comet Assay). For induction of (oxidative) DNA damage, cells were treated with menadione (Md, 6 μM, 1 h in serum-free medium) and isolated by trypsin treatment (*21*). The alkaline single-cell gel electrophoresis (Comet assay) was performed, as described previously (*21*). Briefly, $4 \times 50\,000$ cells were centrifuged, and the pellet was mixed with low melting agarose, applied onto a precoated microscope slide, submitted to lysis, and treated with FPG-enzyme to recognize specific oxidative DNA damage. After DNA unwinding (pH 13.5, 20 min, 4 °C) and horizontal gel electrophoresis (20 min, 25 V, 300 mA), slides were washed, stained with ethidium bromide, and analyzed using a fluorescence microscope (Axioskop 20, filter set 15, Zeiss, Germany) and computerized image analysis (Comet Assay II, Perceptive Instruments, Suffolk, GB), scoring 2×50 cells per slide. DNA migration was calculated as mean tail intensity (TI%: DNA in the comet tail in percent of total DNA). Results were expressed as relative TI in percent of Md-treated control.

Statistics. Results of cell assays are presented as mean ± SD of 3−6 independent experiments, each performed at least in duplicate. Data were analyzed for significant difference ($p < 0.05$) to either oxidant-treated control (Comet assay, DCF assay) or respective solvent control (tGSH determination) by one-sided *t* test.

RESULTS AND DISCUSSION

Phenolic Compounds in Fermented Apple Juice Extracts. Quantification of AE constituents in F-AEs shows a rapid degradation within 6 h fermentation of both, AE03 and AE04 (**Table 1**). In F-AE03 (6 h), residual amounts (% of original AE concentration) were found for Rut (1.0%), Que-3-glucoside (0.9%), Que-3-rhamnoside (1.0%), procyanidin B1 (2.2%), procyanidin B2 (0.3%), Pz (0.1%), and Pt-2′-*O*-xyloglucoside (0.9%). Hydroxycinnamic acid derivatives were not detectable.

Table 1. AE Constituents in 6 h-Fermented Apple Juice Extracts (F-AE (6 h)), Analyzed by HPLC-DAD, in Comparison to AE03 and AE04[a]

compd	AE03[b] (mg/g)	F-AE03 (6 h) (mg/g)	AE04[b] (mg/g)	F-AE04 (6 h) (mg/g)
procyanidin B1	6.2	0.14	n.d.	n.d.
(+)-catechin	2.7	0.01	n.d.	trace
procyanidin B2	18.4	0.06	12.1	n.d.
(−)-epicatechin	17.7	0.02	12.5	n.d.
phloretin-2-xyloglucoside	31.7	0.29	68.9	0.08
phloridzin	78.9	0.1	48.0	0.01
phloretin-2-xylogalactoside	n.d.	n.d.	4.2	n.d.
chlorogenic acid	19.2	n.d.	183.2	0.05
caffeic acid	4.0	n.d.	7.5	0.03
3-coumaroylquinic acid	3.0	n.d.	9.4	0.02
4-coumaroylquinic acid	5.0	n.d.	66.0	n.d.
5-coumaroylquinic acid	3.8	n.d.	39.8	n.d.
coumaroyl-glucose	n.d.	n.d.	11.9	n.d.
p-coumaric acid	4.2	n.d.	2.6	1.5
quercetin-3-rutinoside	49.1	0.51	4.5	n.d.
quercetin-3-galactoside	8.1	n.d.	1.8	n.d.
quercetin-3-glucoside	12.3	0.11	1.5	n.d.
quercetin-3-xyloside	18.1	n.d.	n.d.	n.d.
quercetin-3-arabinoside	3.5	n.d.	n.d.	n.d.
quercetin-3-rhamnoside	25.1	0.25	4.3	n.d.
total polyphenols	311.0	1.48	478.2	1.69

[a] Values are expressed as mg/g AE. n.d. = not detectable (< 0.2 mg/L; equivalent to 0.001 mg/g AE). [b] Taken from our previous publications (*17, 21*) to compare the composition of polyphenols in fermented and original apple juice extracts. Amount of total AE constituents in 24 h-fermented F-AE03 and F-AE04 were 0.1 and 0.002 mg/g, respectively (*17*).

Table 2. Phenolic Degradation Products in 6 and 24 h-Fermented Apple Juice Extracts (F-AEs), Analyzed by HPLC-DAD-ESI-MS[a]

			F-AE03		F-AE04	
compd	RT (min)	$[M - H]^-$ (*m*/*z*)	6 h (mg/g)	24 h (mg/g)	6 h (mg/g)	24 h (mg/g)
phloroglucinol	6.3	125	5.2	9.9	8.7	trace
3,4-dihydroxyphenylacetic acid	11.7	168	0.8	0.9	n.d.	n.d.
dihydrocaffeic acid	15.2	181	6.4	12.0	39.3	76.4
3-(4-hydroxyphenyl)-propionic acid	19.7	165	28.9	53.6	20.9	42.5
quercetin	38.9	301	1.8	n.d.	n.d.	n.d.

[a] Values expressed as mg compound/g original AE. n.d. = not detectable (< 0.2 mg/g original AE). trace: < 0.4 mg/g.

In F-AE04 (6 h), hydroxycinnamic acid derivatives, dihydrochalcones, and catechins were found in trace amounts (<0.1% of original values). Que glycosides were not detectable, probably due to the low concentration of these substances in AE04. In the subsequent 18 h fermentation period, remaining constituents were further degraded, down to <0.1% of initial values, as shown by comparison to the data of the 24 h fermentation (*17*).

The pattern of quantified degradation products (**Table 2**) corresponds well with the presence of respective precursor compounds in the AEs (**Table 1**): Pt glycosides, present in both AEs, are degraded to PG and 4HPPA (**Scheme 1a**), which were found in all fermentation samples. Concentration of PG after 6 h fermentation was increased in F-AE03 and diminished in F-AE04 after 24 h. PG is an intermediate metabolite whose concentration depends on both generation (mainly from Pt and Que) and degradation. Therefore, reaction kinetics of the underlying biotransformations might well vary with respect to the different polyphenol patterns of the AEs. CuA- and CaA esters are predominantly contained in AE04 (320.4 mg/g in AE04 vs 39.2 mg/g in AE03); correspondingly, their degradation products CaA, CuA, and DHCaA (**Scheme 1b**) were mainly

Table 3. Trolox Equivalent Antioxidant Capacity (TEAC) of Fermented Apple Juice Extracts (F-AE03, F-AE04) in Comparison to Original Extracts AE03 and AE04 and to Fermentation Controls (F-Blanks, Fermented without AEs)[a]

	TEAC (mM Trolox)		
fermentation period (h)	F-AE03	F-AE04	F-blank
0 (before fermentation)	4.2 ± 0.1[b]	4.0 ± 0.1[b]	
6	2.7 ± 0.1	3.1 ± 0.1	0.2 ± 0.1
24	2.1 ± 0.0	2.7 ± 0.2	0.3 ± 0.1

[a] Expressed as the mmolar concentration of a Trolox solution, having an antioxidant capacity equivalent to a solution containing 1 mg/mL of AEs or 10% F-AEs (equivalent to a concentration of 1 mg/mL AE). Values are means ± SD from $n = 3-5$ independent experiments. [b] Taken from ref (*21*) for comparison.

found in F-AE04. Que and 3,4DHPAA, both products of Que-glycoside metabolism (**Scheme 1c**), could only be detected in F-AE03. Additionally, the known polyphenol degradation products 3,4-dihydroxybenzoic acid (protocatechuic acid), 3PPA, and 3HPPA (*24*) were identified in all F-AE samples by HPLC-DAD-ESI-MS. 4-Methylcatechol, which had previously been detected in the pig cecum model as an intestinal metabolite of quercetin (*24*), was verified after 24 h fermentation in both F-AEs. The O-methylated compounds HVA (**Scheme 1b**), ferulic acid, and isoferulic acid, which were found to occur in human fecal water and/or urine after consumption of specific polyphenols (ChA, Rut) or polyphenol-rich diet (*9, 10*), were detected in none of the F-AEs. Neither phenolic constituents (*17*) nor degradation products were found in F-blanks (6 and 24 h).

Trolox Equivalent Antioxidant Capacity (TEAC). The antioxidative capacity of the AEs (4.0 and 4.2 mM Trolox) distinctly decreased during fermentation, especially within the first 6 h (**Table 3**). In the subsequent 18 h fermentation period, the antioxidant capacity was further diminished, with F-AEs (24 h) finally reaching 50 and 68% of the original TEAC values. The respective fermentation controls (F-blank (6 and 24 h)) showed only marginal antioxidant capacities ranging at 0.2−0.3 mM Trolox.

Antioxidant capacities of major AE constituents and known degradation products are summarized in **Table 4**. TEAC values of Rut and Pz, representing about 45 and 11% of the known constituents of AE03 and AE04, respectively, were slightly increased after cleavage of the glycoside bond resulting in the formation of Que and Pt. Phenolic acids, known as further degradation products of polyphenols, showed a lower antioxidant capacity, with TEAC values ranging from 1.0−3.0 mM Trolox, compared to the respective parent compounds or aglyca. This indicates a certain loss in radical scavenging efficacy of the apple phenolics during intestinal degradation. In contrast, ChA, the major constituent in AE04, and its degradation products CaA and DHCaA exhibited similar antioxidant capacities (approximately 1 mM Trolox). For 3PPA, derived from dehydroxylation of DHCaA, and QiA, no antioxidant efficacy was detectable, obviously due to the absence of a phenolic structure. In contrast, the antioxidant activity of CuA, formed by metabolism from *p*-coumaroylquinic acids, is rather high (2.9 mM Trolox) as compared to CaA (1.2 mM Trolox). This is in line with previous findings on structure−activity relationship of hydroxylated phenolic acids: hydroxylation in 3,4 position was found to increase antioxidant activity of phenolic acids except for monohydroxylated cinnamic acids, where the catechol moiety was reported to decrease TEAC values (*25*). For CuA (2.2 mM) and 3,4DHPAA (2.2 mM Trolox), slightly lower TEAC values were reported (*25*), whereas similar orders of

Table 4. Trolox Equivalent Antioxidant Capacity (TEAC) of Phenolic Degradation Products in Comparison to the Respective Parent Compounds[a]

AE constituents	degradation products	TEAC (mM Trolox)[b]
rutin		3.7 ± 0.1[c]
	quercetin	4.8 ± 0.1[c]
	3,4-dihydroxyphenylacetic acid	3.0 ± 0.3
	3-hydroxyphenylacetic acid	1.0 ± 0.1
	homovanillic acid	1.7 ± 0.4
phloridzin		3.6 ± 0.4[c]
	phloretin	3.7 ± 0.4[c]
	3-(4-hydroxyphenyl)propionic acid	2.6 ± 0.9
	phloroglucinol	2.1 ± 0.2
chlorogenic acid		1.1 ± 0.3[c]
caffeic acid		1.2 ± 0.2[c]
	dihydrocaffeic acid	1.1 ± 0.3
	3-phenylpropionic acid	0.0 ± 0.0
coumaroyl-quinic acids		n.a.[d]
	p-coumaric acid	2.9 ± 0.3
	(−)-quinic acid	0.0 ± 0.0

[a] Values are means ± SD from $n = 3-5$ independent experiments. [b] Antioxidant capacity of a Trolox solution having an antioxidant capacity equivalent to 1.0 mM solution of the test sample. [c] Taken from ref (*21*) for comparison. [d] n.a. = not analyzed.

phenolic acid antioxidant activity were obtained by different radical scavenging assays (*25, 26*).

Taken together, the TEAC of the AEs and their constituents is diminished during intestinal degradation. However, the F-AEs and the majority of the degradation products tested still show distinct antioxidant capacity (1−3 mM Trolox).

Cellular ROS Level. Modulation of TBH-induced, cellular ROS level in Caco-2 cells after 24 h incubation with fermented apple juice extracts (0.0015−0.5%) is shown in **Figure 1**. With F-AE03 (6 and 24 h), a significant, concentration dependent decrease was observed in the higher concentration range (**Figure 1a**). Maximal reduction of ROS level was evoked by 0.5% F-AE03 (corresponding to 50 mg/mL unfermented AE). In lower concentrations (<15 mg/mL), unfermented AE03 was similarly effective in reducing cellular ROS level compared to the F-AE03; concentrations of AE03 exceeding 15 mg/mL, however, were less efficient than the F-AEs. F-AE04 (6 and 24 h) distinctly reduced cellular ROS (**Figure 1b**) down to one-third of TBH-control (with 0.5% F-AE) whereas the unfermented AE04 showed only moderate effects, decreasing TBH-control by maximally 30%. The fermentation controls (F-blanks), which were found to contain neither AE polyphenols nor degradation products, also reduced the cellular ROS level, F-blank (24 h) being more effective than F-blank (6 h), especially at low concentrations (**Figure 1a**,**b**). F-AEs (6 h) but not F-AEs (24 h) were clearly more effective than the respective F-blank; the data suggest that phenolic constituents/degradation products account for up to 25% of F-AE (6 h) activity. Most effects, however, can be ascribed to fecal components such as short chain fatty acids (SCFA), generated during fermentation of dietary fibers, sugars and also polyphenols (*6, 17*). These compounds have been quantified in substantial amounts in both, F-AEs (24 h) (28.9−35.4 mmol/L) and F-blank (24 h) (11.4 mmol/L) (*17*). The increase of SCFA formation in the course of fermentation corresponds well with the decrease of cellular ROS level by 6 and 24 h fermented samples.

The decrease of cellular ROS level by individual phenolic degradation products in comparison to the parent compounds ChA and Que is shown in **Figure 2**. The degradation products 3,4DHPAA and PG were less efficient than the precursor compound Que

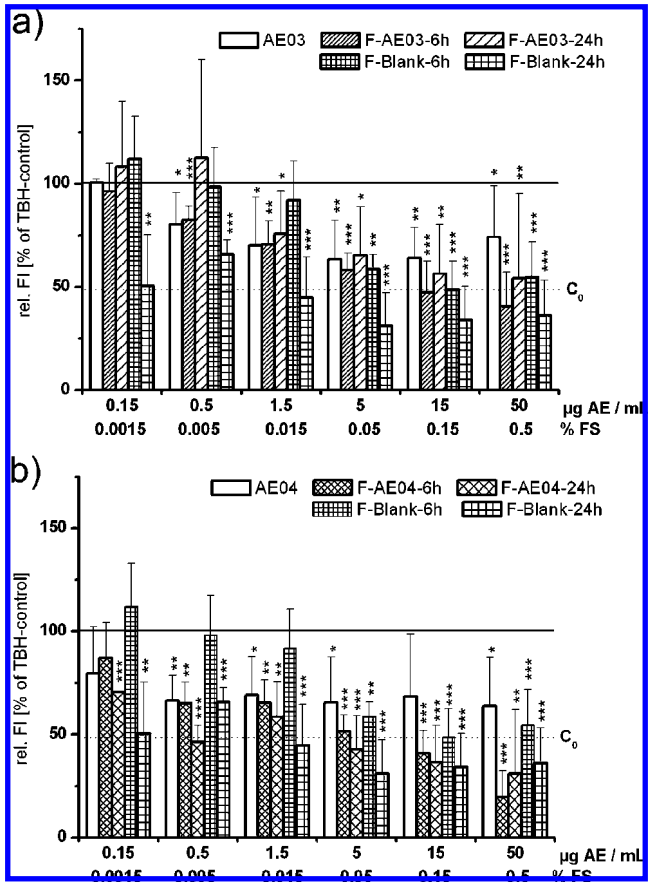


Figure 1. Modulation of TBH-induced cellular ROS level in Caco-2 cells after 24 h incubation with fermented (F-) and original AEs: (**a**) AE03; (**b**) AE04. F-blanks are included in both pictures; $n = 3-5$ (mean ± SD); significantly lower than TBH-treated solvent control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; C_0 solvent control, without TBH treatment.

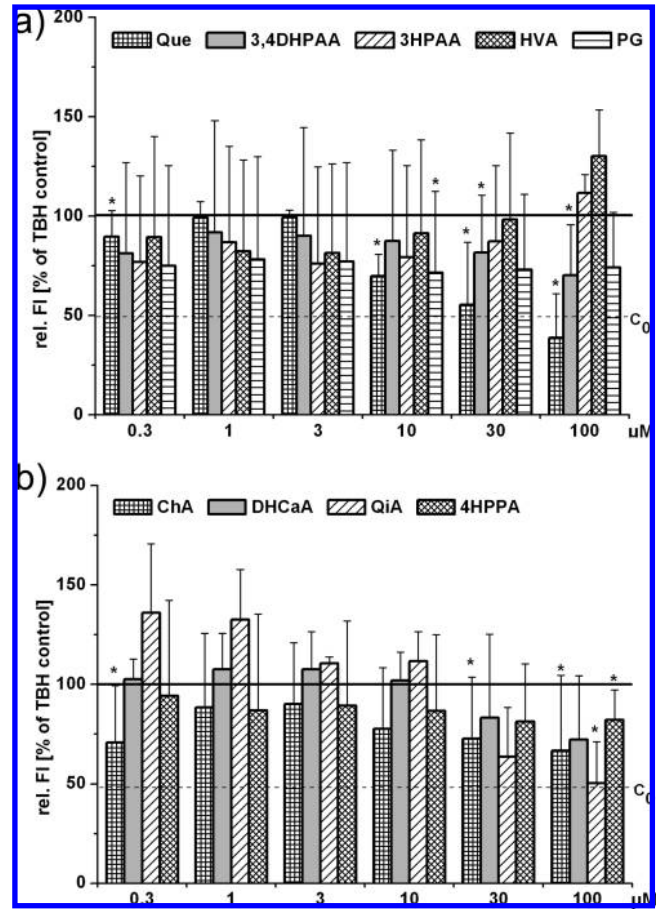


Figure 2. Modulation of TBH-induced cellular ROS level in Caco-2 cells after 24 h incubation with phenolic degradation products from (**a**) flavonoids and (**b**) hydroxycinnamic acids, dihydrochalcones; $n = 3-5$ (mean ± SD); significantly lower than TBH-treated solvent control: * $p < 0.05$; C_0 solvent control, without TBH treatment.

but still distinctly antioxidant (**Figure 2a**). Thus, PG, a major phenolic component in F-AE03, might well contribute to the antioxidant potential of the fermented extract. In contrast, 3HPAA and HVA showed no such activity at 0.3−100 μM. Reduction of cellular ROS level by hydroxycinnamic acid and dihydrochalcone degradation products is shown in **Figure 2b**. A significant reduction was observed for QiA and 4HPPA at >30 μM whereas the parent compound ChA significantly reduced cellular ROS level already at >10 μM. A slight but not significant decrease in ROS level was also obtained with DHCaA (≥30 μM), a major phenolic in both F-AEs (*17*). Further degradation products, CuA and 3PPA, showed no antioxidant activity at the concentration range 0.3−100 μM (data not shown).

The presence of catalase (100 U/mL) in the incubation medium did not significantly alter the ROS-reducing capacity of Que (0.3−100 μM, data not shown), a known peroxide generator in cell culture media (*18, 19*). Similarly, no modulation of CaA, F-AE, and F-blank results was evoked by addition of catalase. This strongly suggests that our results on antioxidant effectiveness of phenolic constituents/degradation products are not impaired by extracellular peroxide generation.

Cellular Glutathione Level. Indications for a slight increase of total glutathione level (tGSH = GSH + GSSG) in Caco-2 cells were obtained with F-AE03 at 0.15 and 0.5%, the highest concentrations tested (**Figure 3a**). In contrast, F-AE04 as well as F-blanks clearly caused a decrease of glutathione level down to about 80% of solvent control (**Figure 3b**,**c**). This suggests a depletion of cellular glutathione level by fecal components, which could be due to induction of glutathione-S-transferases by SCFA (*27*) or a conjugation of phenolic acids with glutathione (*28*). The tGSH elevation specifically obtained with F-AE03 can be ascribed to degradation products of Que glycosides, which compensate for the depletion caused by fecal components.

In all experiments, no significant change of GSSG and GSH/GSSG-ratio was observed, suggesting that the GSH/GSSG redox system of Caco-2 cells was not distinctly modulated by F-AEs or F-blanks.

Oxidative DNA Damage. Menadione-induced oxidative DNA damage in Caco-2 cells was slightly diminished by F-AE03 (6 h) and F-blank (6 h) (**Figure 4**) but not with F-AE04 (6 h) or the 24 h fermentation supernatants (data not shown). Since the F-AE03 (6 h) reduced oxidative DNA damage less efficiently than the previously studied original extract AE03 (*21*), the DNA-protecting effect of AE03 appears to be continuously decreased during the fermentation process.

Taken together, our results give strong evidence that the antioxidant potential of polyphenolic apple juice extracts survives fermentation by human fecal microflora, although it is diminished by degradation processes. This is reflected by the fermentation-mediated decline of cell free antioxidant capacity of the AEs down to 50% and by a concomitant reduction of protecting activity against cellular ROS and DNA damage in Caco-2 cells. Similarly, degradation products identified in F-AEs mostly were less antioxidant (TEAC and cellular ROS level) than the respective AE constituents. A loss of preventive efficacy during 24 h fermentation of AEs was also reported by Veeriah

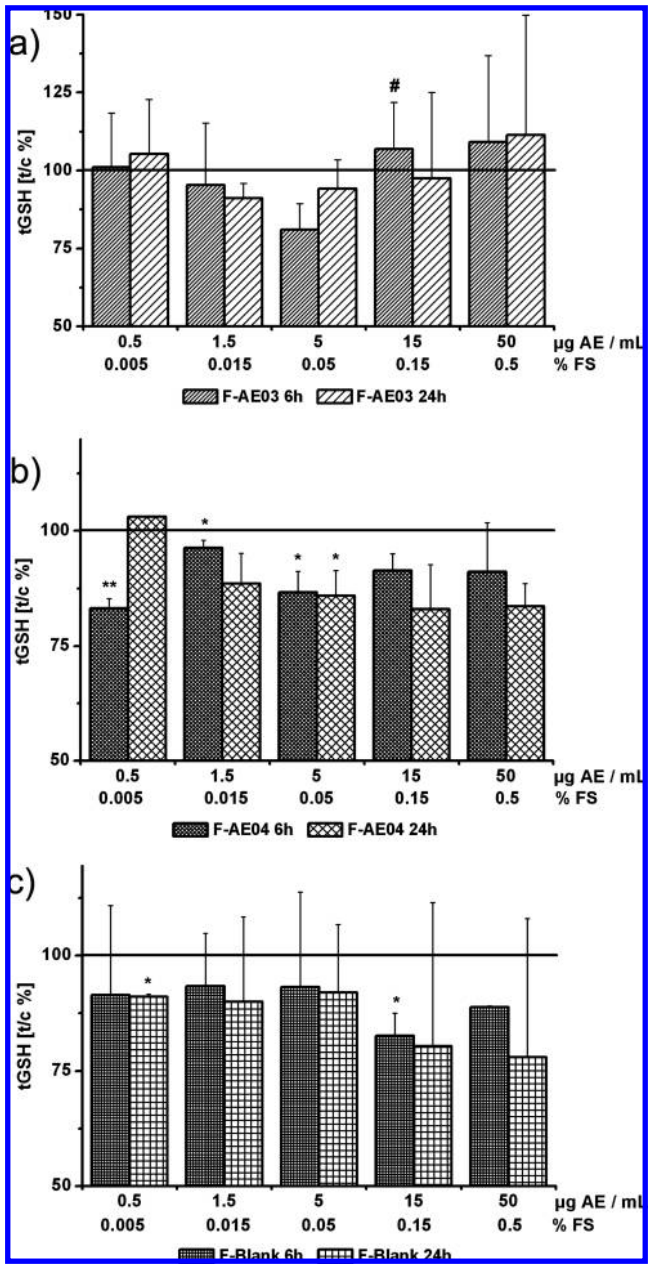


Figure 3. Modulation of total glutathione (tGSH) in Caco-2 cells after 24 h incubation with fermented AEs: (**a**) F-AE03; (**b**) F-AE04; (**c**) F-blanks. $n = 3-5$ (mean ± SD); significantly lower than solvent control (= 100%): * $p < 0.05$, ** $p < 0.01$; significantly lower than respective F-blank: # $p < 0.05$.

et al., using the marker antiproliferative activity in the colon cancer cell lines LT-97 and HT-29 (*17*).

The capacity of AE03 to elevate the tGSH level however was found to be unchanged or even raised by fermentation. This specific effect of F-AE03 is supposed to result from its high amount of Que glycosides, which are cleaved by colonic metabolism. This is in line with the findings that Que, in contrast to its glycoside Rut, elevates the glutathione level (*21*) and induces transcription of γ-glutamyl cysteine ligase (catalyzing the rate limiting step of glutathione synthesis) in Caco-2 (*29*) and other cell lines (*30*).

Since more than 99% of the original polyphenolic AE constituents were found degraded within the first 6 h of fermentation, the observed antioxidant activity of F-AEs can be ascribed to phenolic fermentation products such as 4HPPA or PG, which have shown distinct efficiency to reduce cellular

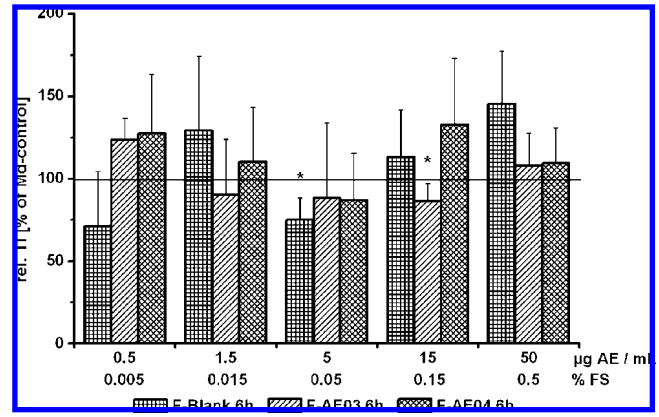


Figure 4. Modulation of menadione (Md)-induced oxidative DNA damage in Caco-2 cells after 24 h incubation with 6 h-fermented AEs and F-blank; $n = 3-5$ (mean ± SD); significantly lower than Md-control: * $p < 0.05$; DMSO control: mean TI = 1.4%/Md-control: mean TI = 5.1%.

ROS and to scavenge free radicals. We assume that the antioxidant activities of AE polyphenols and metabolites, which we observed in Caco-2 cells, also protect the colon from oxidative stress and thus might contribute to prevent ROS-mediated diseases.

Nonphenolic degradation products such as SCFA exhibit no direct antioxidant capacity; however, decrease of oxidative DNA damage in HT-29 cells and increase of catalase activity by butyrate have been reported (*31, 32*). Since the concentration of SCFA increases during fermentation (*17*) and no effect of 24 h fermented samples on oxidative DNA damage were observed, SCFA are not supposed to play a major role in DNA protection by F-AEs. No effects of SCFA on glutathione level and activities of glutathione peroxidase and superoxide dismutase were reported (*32*), confirming that modulation of GSH by F-AE03 is specifically due to phenolic degradation products and not to SCFA.

In conclusion, fermented apple juice extracts exhibit antioxidant capacity, which at least in part can be ascribed to phenolic degradation products rather than to nonphenolic fecal components. This is supported by substantial antioxidant activities of individual phenolic acids and other phenolic derived metabolites.

ABBREVIATIONS USED

3,4DHPAA, 3,4-dihydroxyphenylacetic acid; 3HPAA, 3-hydroxyphenylacetic acid; 3PPA, 3-phenylpropionic acid; 3HPPA, 3-(3-hydroxyphenyl)propionic acid; 4HPPA, 3-(4-hydroxyphenyl)propionic acid; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); AEs, apple polyphenol extracts; AE03, apple pomace extract, harvested 2003; AE04, apple extract, harvested 2004; CaA, caffeic acid; ChA, chlorogenic acid; CuA, *p*-coumaric acid; DAD, diode array detection; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DHCaA, dihydrocaffeic acid; DMEM, Dulbecco's modified eagle medium; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ESI, electrospray ionization, F-AEs, fermented apple polyphenol extracts; FPG, formamidopyrimidine-DNA-glycosylase; FS, fermentation supernatant; GSR, glutathione reductase; HPLC, high-performance liquid chromatography; HVA, homovanillic acid; Md, menadione; MS, mass spectrometry; PG, phloroglucinol; Pt, phloretin; Pz, phloridzin; QiA, quinic acid; Que, quercetin; ROS, reactive oxygen species; Rut, rutin; SCFA, short chain fatty acids; SSA, 5-sulfosalicylic acid; TBH, *tert*-butylhydroperoxide; TEA, triethanolamine.

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