Of the Major Phenolic Acids Formed during Human Microbial Fermentation of Tea, Citrus, and Soy Flavonoid Supplements, Only 3,4-Dihydroxyphenylacetic Acid Has Antiproliferative Activity[1,2]

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ABSTRACT Dietary flavonoids are poorly absorbed from the gastrointestinal tract. Colonic bacteria convert flavonoids into smaller phenolic acids (PA), which can be absorbed into the circulation and may contribute to the chemopreventive activity of the parent compounds. The purpose of our study was to determine whether flavonoids from green and black tea (GT, BT), citrus fruit with rutin (CF+R) and soy (S) supplements exposed to the same conditions in a dynamic in vitro model of the colon (TIM-2) will form the same phenolic acid products of microbial metabolism. About 600 mg of flavonoids from GT, BT, CF+R and S extracts were infused at $t = 0$ and 12 h into the TIM-2. Samples from the lumen and dialysate were collected at $t = 0,4,8,12,16,24$ and 28h. The flavonoid and PA concentrations were measured by HPLC and GC-MS. GT, BT, and CF+R formed 3-methoxy–4-hydroxyphenylacetic acid (3M4HPAA), 4-hydroxyphenyl acetic acid (4HPAA), 3,4-dihydroxyphenylacetic acid (3,4DHPAA), and 3-(3-hydroxyphenyl) propionic acid (3,3HPPA). BT flavonoids were also metabolized to 2,4,6-trihydroxybenzoic acid (2,4,6THBA) and CF+R flavonoids to 3-(4-hydroxy–3-methoxyphenyl) propionic acid (3,4H3MPPA), 3-hydroxyphenyl acetic acid (3HPAA) and a small amount of hippuric acid. After S infusion, we found 3M4HPAA and 4HPAA only. Among these phenolic acids, only 3,4DHPAA exhibited antiproliferative activity in prostate and colon cancer cells. 3,4DHPAA was significantly ($P < 0.005$) more inhibitory in colon cancer cells (HCT116) compared with an immortalized normal intestinal epithelial cell line (IEC6). In summary, fermentation by intestinal microbes of GT, BT, C+R, and S flavonoids resulted in the conversion to the same major phenolic acids. J. Nutr. 136: 52–57, 2006.

KEY WORDS: • *flavonoids* • *colonic fermentation* • *antiproliferative activity* • *phenolic acids*

The largest group of ingested plant-derived polyphenolic compounds are called flavonoids (**Fig. 1**) (1). Evidence from cell culture, animal, and epidemiologic studies indicates that flavonoids from green tea, fruits and vegetables, and soy may have cancer preventive potential (2). Many flavonoids act as antioxidants, but also exhibit biological activities including inhibition of proliferation and angiogenesis, modulation of signal transduction, and phytoestrogenic activity (3,4). The major dietary sources of flavanones (naringenin, hesperidin) are grapefruits and oranges. The flavan-3-ols [epicatechin (EC),[4] epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG) and theaflavins (THF)] are derived from tea, grape skin, and chocolate. The flavonols (quercetin, kaempherol, myricetin) come primarily from onions, apples, broccoli, spinach, and kale. Soy is the primary source of the isoflavones (genistein and daidzein), and anthocyanins (cyanidin, delphidin) are derived from berries (1,5).

[1] Presented in part at Experimental Biology 05, April 2005, San Diego, CA [Henning SM, Krul C, Seeram NP, Niu Y, Liu Y, Heber D. Colonic metabolism of flavanols from green and black tea studied using an in vitro large intestinal model (abstract). FASEB J. 2005;19(4): A415].

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[4] Abbreviations used: BT, black tea; CF+R, citrus fruit with rutin; 3,4DHBA, 3,4-dihydroxybenzoic acid; 3,4DHPAA, 3,4-dihydroxyphenylacetic acid; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; GT, green tea; IC_{50}, concentration required to suppress cell growth by 50%; 4HBA, 4-hydroxybenzoic acid; 3,4H3MPPA, 3-(4-hydroxy-3-methoxyphenyl) propionic acid; 3HPAA, 3-hydroxyphenylacetic acid; 4HPAA, 4-hydroxyphenylacetic acid; 3,3HPPA, 3-(3-hydroxyphenyl) propionic acid; 3M4HBA, 3-methoxy-4-hydroxybenzoic acid/ vanillic acid; 3M4HPAA, 3-methoxy-4-hydroxyphenylacetic acid/ homovanillic acid; S, soy; 2,4,6THBA, 2,4,6-trihydroxybenzoic acid; THF, theaflavin; THF3G, theaflavin-3-monogallate; THF3′G, theaflavin-3′-monogallate; THF33′GG, theaflavin-3,3′-digallate.

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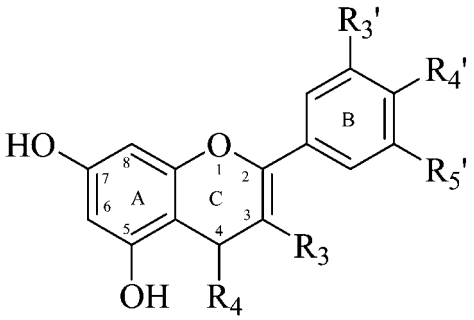

FIGURE 1 General structure of flavonoids. For flavanones, flavonols, and isoflavones R_4 = 4-oxo, R_3 = H; for hesperitin $R_{4'}$ = methoxo; for flavan-3-ols R_4 = H, R_3 = OH or gallate.

Except for flavan-3-ols from tea, flavonoids occur in nature mainly in the form of glycosides (1). The structural diversity of flavonoids influences their intestinal absorption. Only ~2–15% of the flavonoids ingested are absorbed in the upper gastrointestinal tract. Flavonoids can reach the colon in 2 ways, i.e., either nonabsorbed flavonoids (-glycosides) pass through the small intestine, or flavonoids that were initially absorbed are subsequently excreted as conjugates in bile and pass through the small intestine. In the colon, bacterial glycosidases, glucuronidases, and sulfatases remove all remaining glycosides, glucuronides, and sulfates from the flavonoid molecule. The flavonoid aglycon can undergo further bacterial metabolism to ring fission products such as valerolactones (6,7), and a wide array of low-molecular-weight phenolic acids (8). It was suggested that these simple phenolic compounds can be absorbed into the blood stream and excreted in the urine; they may contribute to the health benefits of flavonoid consumption. Rechner et al. (8) demonstrated increased urinary and plasma levels of simple phenolic acids such as 3-hydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid after the ingestion of a flavonoid-rich meal. However, our knowledge is limited to which metabolites are formed and absorbed in the colon and which metabolites are formed during transition into the circulation and by metabolism in organs such as liver and kidney. Based on the structural similarity of flavonoids, we hypothesized that flavonoids from different food sources are transformed to the same phenolic acid products. Therefore the purpose of this study was to characterize and quantitate phenolic acid products formed during colon digestion from green tea (GT), black tea (BT), citrus+rutin (CF+R) or soy (S) flavonoids digested under the same conditions using pooled human colonic microbiota.

We used a dynamic in vitro model of the large intestine (TIM-2; TNO Quality of Life) that simulates colonic fermentation by the intestinal microbiota (9–11). This computer-controlled system mimics the human physiologic processes of the large intestine by maintaining standardized conditions such as pH in the lumen, composition and rate of secretion, delivery of a substrate from the ileum, peristaltic mixing, and transport of the intestinal contents (**Fig. 2**). It contains a complex anaerobic microbiota of human origin. During the initial validation of the system, the compatibility and stability of the pooled microbiota maintained in a fermentor before inoculation were confirmed in a previous study by comparison with fresh fecal samples (11). High densities of microorganisms, comparable to those found in the colon in vivo, are achieved by absorption of water and dialysis of metabolites through hollow-fiber membranes inside the reactor compartment (11) . The model offers the opportunity to compare the digestion of different ingested products under identical and standardized controlled conditions using the same pool of human microbiota.

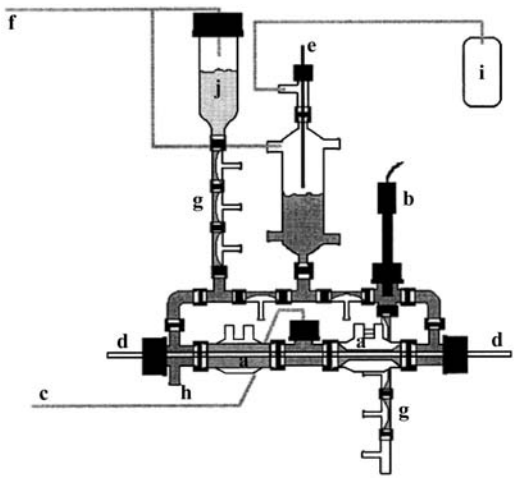

FIGURE 2 Schematic representation of the in vitro model of the proximal colon (TIM-2; 17): a. peristaltic compartments; b. pH-electrode; c. pH control by secretion of NaOH; d. hollow-fiber semipermeable membranes; e. level-sensor; f. N_2 gas inlet; g. inlet and outlet valves; h. sampling-port; i. gas collection bag; j. ileal delivery medium.

MATERIALS AND METHODS

Chemical products. Rutin, quercetin, eriocitrin, eriodictyol, naringin, naringenin, hesperidin, hesperitin, kaempherol, genistin, genistein, daidzin, daidzein, and equol were purchased from Indofine Chemical, theaflavins from Wako Chemicals USA, 3-hydroxyphenylacetic acid (3HPAA), 4-hydroxyphenylacetic acid (4HPAA), 3-methoxy-4-hydroxyphenylacetic acid/ homovanillic acid (3M4HPAA), 3-methoxy-4-hydroxybenzoic acid (3M4HBA), 3,4-dihydroxyphenylacetic acid (3,4DHPAA), cinnamic acid, 4-hydroxybenzoic acid (4HBA), 3,4-dihydroxybenzoic acid (3,4DHBA), hippuric acid, *p*-coumaric acid, and ferulic acid were available from Sigma Chemical, 3-(4-hydroxy–3-methoxyphenyl) propionic acid (3,4H3MPPA) was purchased from Lancaster Synthesis, and 3-(3-hydroxyphenyl) propionic acid (3,3HPPA) from Transworld Chemicals; phloroglucinol from MP Biomedicals; 4-methoxysalicylic acid from Fisher Scientific; and 2,4,6-trihydroxybenzoic acid (2,4,6THBA) was purchased from Fluka Chemika. All chemicals were of analytic grade (>99%).

The dynamic In Vitro model of the large intestine. This model was described previously in detail (10,12). The model is patented in Europe (no. 0642382) and the United States (no. 5,525,305). Dialysis fluid was pumped through semipermeable hollow-fiber membranes, placed inside the lumen of the model, to remove water and microbial fermentation products. The flow of the dialysis fluid through the hollow fiber membrane was 1 mL/min. The amount of chyme in the system was measured by a volume sensor and kept constant at 110 mL by the removal of additional fluid through the dialysis system. The standardized human microbiota consisted of fecal samples derived from 10 healthy volunteers (19–35 y old). The mixture was cultured in a fermentor and stored at −80°C. These aliquots, mixed with the artificial ileal delivery medium, were used as inocula for the TIM-2 model. The complex composition of the ileal delivery medium simulating the material passing the ileocecal valve in humans was described by Gibson et al. (11,13). The environment in the model is kept strictly anaerobic by flushing with gaseous nitrogen to allow the growth of the microbiota. This experimental system was validated with respect to microbial composition and activity and for reproducibility using pooled human microbiota (10,14). Therefore, the current experiments were performed in single runs using the same human microbiota pool for all 5 experiments.

Flavonoid powder. GT and BT were brewed using Japanese Green Tea leaves and Twinings English Breakfast tea bags, respectively. The teas were chosen because of their high content of flavanols. Several batches of 20 g tea/L boiling water were brewed, frozen, and freeze-dried in a Lyph-lock 6 freeze-dry system (Labconco). The flavonoid content of the following 6 different commercial citrus supplements was screened: *1*) Citrus Bioflavonoid Caps, Twin Laboratories; *2*) Bioflavonoid Caps, Now Foods; *3*) Citrus Bioflavonoid complex, Solgar Vitamin and Herb;

4) Citrus Bioflavonoids complex 1000, General Nutrition; and 5) Citrus Bioflavonoids plus Hesperidin, Natural Factors; and 6) Citrus Bioflavonoids, Country Life). In addition the following 4 soy flavonoid products were screened: *1*) Perfect Soy, Metagenics; *2*) Soy Choice, Vitanica; *3*) PhytoSoya, Arkopharma, LLC; and *4*) Isoflavones, Solgar Vitamin and Herb). Capsules were ground to a powder, extracted with water:methanol (70:30, v:v), concentrated and freeze dried. The first extract and the final product were analyzed in our laboratory. The supplements with the highest flavonoid content (Citrus Bioflavonoid Caps and Perfect Soy, Metagenics) were used for the experiment. The flavonoid content was concentrated 2- to 7-fold over the original commercial supplement and final flavonoid composition determined.

Experimental Design. The same pooled, standardized human microbiota was used in all 5 (4 extracts and 1 control) experiments. At the start of the experiment, the model was inoculated with ~30 mL of the standard, cultivated fecal microbiota. After adaptation of the human microbiota for 16 h (10) a total of 1.5 g of the different concentrated flavonoid supplements dissolved in methanol:water mixture (50:50, v/v) was added to the system at $t = 0$ and 12 h. Samples from the luminal and dialysis fluids were collected before and at 4, 8, 12, 16, 24, and 28 h after infusion of the flavonoid solutions; 2.5 and 10 mL of these samples were used for chemical analysis, respectively. In the control experiment, only the solvent of the extracts was added. Lumen samples at the start ($t = 0$ h) and at the end of the experiment ($t = 28$ h) were used to evaluate the stability of the microbiota. The following bacteria were enumerated: *Bifidobacterium, Bacteroides, Clostridium*, *Lactobacillus, Enterobacteriaceae* and *Enterococcus* (12). Additionally the concentration of short-chain fatty acids (SCFA) was determined to check the activity of the microbiota at each time point (14). The following SCFA were determined by GC (Varian Chrompack CP9001): acetate, propionate, butyrate, valerate, isobutyric acid, and isovalerate as described by van Nuenen et al. (15). In our experience, the analytical variation is very small; therefore, samples were analyzed without replicates. At each time point, the amount of flavonoids or phenolic acids was determined in the dialysate and the lumen samples. The total amount of a compound present at a certain time point was calculated by adding the amount determined in the dialysate and in the lumen, corrected for the amount of a compound already present in the lumen at the previous time point. The following equation was used:

Total amount of compound Y = $\mathrm{Conc_{DTn}} - \mathrm{Conc_{LTn-1}} + \mathrm{Conc_{LTn}} + \mathrm{Conc_{LsampleTn-1}} + \mathrm{Conc_{CumTn-1}}$ with Conc = concentration, D = dialysate, T = time point, L = lumen, Tn−1 = previous time point, Lsample = amout of sample removed from the lumen for testing, and Cum = cumulative amount.

Analysis of flavonoids. The flavonoid analysis was performed by HPLC using a C18 Alltima column with inside diameter of 53 mm × 7 mm, particle size of 5 μm (Alltech). The column was eluted at 25°C with a linear gradient from 100% buffer A (75 mmol/L citric acid/25 mmol/L ammonium acetate) to 100% buffer B (75 mmol/L citric acid/25 mmol/L ammonium acetate:acetonitrile, 50:50) over 35 min at a flow rate of 1 mL/min. The Agilent 1100 Series quaternary pump solvent delivery system, autosampler, and Chemstation Software 9.01 (Agilent Technology) were used. The eluent was monitored at 254, 260, and 320 nm using an Agilent 1100 diode array detector (16).

Analysis of phenolic acids. Samples were extracted with ethylacetate (1:5, v:v). 3-Hydroxycinnamic acid was added as an internal standard. The volume of the supernatant was reduced in a Savant SC100 speed-vacuum centrifuge (250 × *g*; 20 min). The remaining sample was frozen to remove any remaining water-based layer. The remaining solution was reextracted with ethylacetate, mixed with silylation solution, and incubated at 60–70°C for 1 h. A Restek Rtx-5 stabilized phase fused silica column (30 m × 0.25 mm i.d., 0.25μm film) was used to carry out the separation (17). To characterize the trimethylsilyl derivatives by GC-MS the Thermoquest Trace2000 GC-MS system capable of electron and chemical ionization modes was utilized. The following phenolic acid standard compounds were used for the GC-MS analysis of the lumen and dialysis fluids as potential fermentation products of the infused flavonoids: 3M4HBA, 3M4HPAA, ferulic acid, hippuric acid, 3,4DHPAA, 3,4DHBA, 3HPAA, 4HBA, 4HPAA, phloroglucinol, 4-methoxysalicylic acid, *p*-coumaric acid, 2,4,6THBA, 3,3HPPA and 3,4H3MPPA. Analyses were performed in duplicate.

Antiproliferative activity of phenolic acids. The LNCaP prostate cell line, and the colonic cell lines HCT116 (cancer cell line) and IEC6 (normal intestinal epithelial cell line) were purchased from ATCC. LNCaP cells were maintained in RPMI 1640 medium (VWR Scientific), supplemented with 10% FBS, 10^5 U/L penicillin, and 100 μg/L streptomycin. HCT116 cells were grown in McCoy's 5A modified medium (ATCC) supplemented with 10% FBS, 10^5 U/L penicillin, and 100 μg/L streptomycin, and IEC6 cells were cultured in DMEM medium (ATCC) supplemented with 10% FBS, 4 mmol/L L-glutamine, 10^5 U/L penicillin, 100 μg/L streptomycin, and 100 U/L bovine insulin (Sigma Chemical) according to ATCC protocol. Cells were grown at 37°C in a humidified atmosphere supplemented with 5% CO_2 in air. The doubling time for HCT116, IEC6, and LNCaP was 42, 50, and 36 h, respectively. Then, 3–5 × 10^3 cells/well were seeded in 96-well dishes. After 24 h, the medium was replaced with that containing the appropriate phenolic acid concentration or ethanol vehicle. Cells were incubated at 37°C for another 24 h before cell proliferation was determined. Cell proliferation was quantitated using the CellTiter-Glo™ Assay (Promega), which determines the number of viable cells in culture based on quantitation of the amount of ATP present; this in turn signals the presence of metabolically active cells. Experiments were performed in 3–4 replicates.

Statistical Analysis. PRISM statistical analysis software package version 4 (GraphPad Software) was used for statistical analyses. Data are expressed as means ± SD. The antiproliferative activity of HCT116 and IEC6 cells was compared at each concentration using Student's *t* test. Bacteria counts were compared between time 0 and 28 h for each of the 4 flavonoid interventions and the control using Student's *t* test. The concentration required to suppress cell growth by 50% (IC_{50}) was calculated using dose-response data analysis according to the PRISM statistical analysis software package version 4 (GraphPad Software).

RESULTS

The GT supplement used contained a total of 691 mg of polyphenols: 358, 64, 207, and 62 mg/total infusion of EGC, EC, EGCG, and ECG, respectively. The BT supplement contained 505 mg of tea polyphenols: 48, 20, 232, 101, 25, 45, 0.5, and 33 mg/total infusion of EGC, EC, EGCG, ECG, THF; THF-3-monogallate (THF3G); THF-3′-monogallate (THF3′G); and THF-3,3′-digallate (THF33′GG), respectively. The CF+R supplement contained a total of 807 mg of flavonoids. It had the following composition: 284, 430, 88, and 4.4 mg of rutin, naringin, hesperidin and eriodictyol, respectively. The S supplement contained a total of 553 mg of the following flavonoids: 341, 193, 13, and 5 mg/total infusion of daidzin, genistin, daidzein, and genistein, respectively. The amounts were chosen based on the sensitivity of the analytical method to determine phenolic acid composition of the lumen and dialysate as well as on representing a possible dietary intake. The amount of flavonoids used for the total infusion was 505–807 mg. A serving of 250 mL of GT contains ~150–200 mg of polyphenols. The hesperidin and naringenin glycoside concentration of orange or grapefruit juice is 380–800 mg/L, and soybeans contain ~150 mg isoflavones/100 g. Therefore the amount of flavonoid used in the present study would represent 3–5 servings of tea (750–1250 mL), 1 L of juice, or 370 g of cooked soybeans.

Green tea fermentation. The GT powder was produced using a Japanese green tea with a high amount of EGC. The maximum cumulative of EGCG, EC and ECG amount determined in the lumen and dialysate combined was 4, 11, 1% of the concentration infused, respectively, whereas EGC comprised 34% of the original amount infused (**Fig. 3***A*). The fermentation products determined after the infusion of GT were 3M4HPAA, 4HPAA, 3,3HPPA, 3,4DHPAA, and 2,4,6THBA (Fig. 3*E* and **Table 1**). 3M4HPAA concentration remained high at 28 h, whereas 4HPAA and 3,4DHPAA reached a maximum at 16 h and decreased to ~4–7 μmol/L of digestion volume at 28 h (Fig. 3*E*).

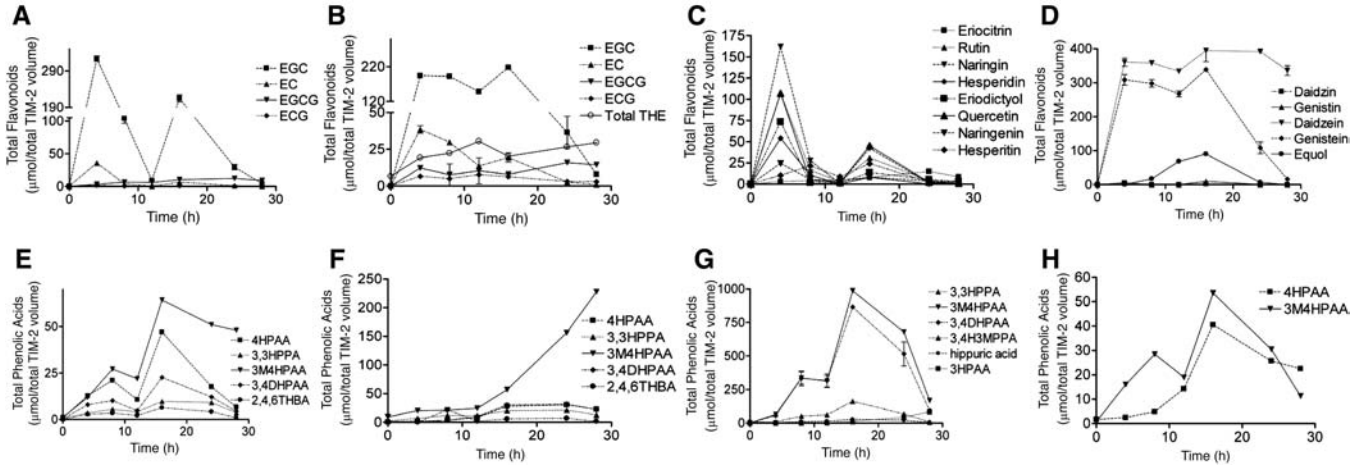

FIGURE 3 Concentrations of EGC, EC, EGCG, ECG, theaflavins, 4HPAA, 3,3HPPA, 3M4HPAA, 3,4DHPAA, 2,4,6THBA, 3,4H3MPPA, hippuric acid, and 3HPAA in lumen and dialysate combined at time 0–28 h after infusion of GT (*A* and *E*); BT (*B* and *F*); CF+R (*C* and *G*); and S (*D* and *H*). Values are means ± SD; $n = 3$ (flavonoids) or 2 (phenolic acids).

Black tea fermentation. After BT infusion into the in vitro colon model, the maximum cumulative amount of EGC in the lumen and dialysate increased to 224% of the original amount infused (Fig. 3*B*), whereas EGCG, EC, and ECG decreased to 2, 60, and 4% of the tea powder content, respectively (Fig. 3*B*). The sum of the 4 theaflavins analyzed in the lumen and dialysate did not change (Fig. 3*B*). The main fermentation product was 3M4HPAA and smaller amounts of 4HPAA, 3,3HPPA, 3,4DHPAA, and 2,4,6THBA were present after BT infusion (Fig. 3*F* and Table 1). 3M4HPAA had a kinetic pattern similar to that for GT. The lumen content of 3M4HPAA after BT consumption continued to increase beyond 16 h (Fig. 3*F*). No hippuric acid was present in the lumen or dialysate.

Citrus+rutin fermentation. The flavonoids in the CF+R powder were almost all in the glycoside form. After the infusion of CF+R supplement into the in vitro colon model, the flavonoid glycoside concentration (eriocitrin, rutin, naringin, hesperidin) decreased rapidly and aglycones (eriodictyol, quercetin, naringenin, hesperitin) increased (Fig. 3C). The fermentation products were 3M4HPAA, 3,4DPAA, 3,3HPPA, 3,4H3MPPA, 3HPAA, hippuric acid, and 4HPAA (Fig. 3G and Table 1).

Soy fermentation. Soy flavonoids in the soy powder infused into the lumen were almost all in the glycoside form (daidzin, genistin). After the infusion, the glycosides were converted to the aglycone form (daidzein, genistein) at 4 h. Daidzein and genistein remained at a high concentration for 16 h; only at 28 h did genistein decrease to 5% of the 4-h concentration, whereas daidzein did not decrease (Fig. 3*D*). Equol was detected starting at 6 h, with a maximum at 16 h. 3M4HPAA and 4HPAA were the major phenolic acids produced (Fig. 3*H* and Table 1).

TABLE 1

Phenolic acid composition of the lumen and dialysate combined after green tea, black tea, citrus+rutin and soy infusion[1]

	GT	BT	C+R	S
	% of total phenolic acids			
3M4HPAA	49.0 ± 0.2	61.9 ± 0.2	44.2 ± 0.9	55.6 ± 0.2
4HPAA	25.3 ± 0.2	11.3 ± 0.06	0.5 ± 0.004	44.4 ± 0.2
3,4DHPAA	13.5 ± 0.1	11.9 ± 0.07	37.3 ± 1.2	—[2]
3,3HPPA	7.8 ± 0.08	10.3 ± 0.007	7.9 ± 0.2	—[2]
2,4,6THBA	4.3 ± 0.05	4.5 ± 0.02	—[3]	—[3]
3HPAA	—[2]	—[3]	2.4 ± 0.05	—[3]
Hippuric acid	—[2]	—[2]	1.5 ± 0.02	—[2]
3,4H3MPPA	—[2]	—[3]	6.3 ± 0.1	—[2]

[1] Values are means ± SD, $n = 2$.
[2] Value contributes <0.5% to total.
[3] Below detection limit of 0.5 μmol/L.

Control fermentation. To serve as a control, the same volume of solvent mixture was infused into the lumen. Less that 8 μmol/total TIM-2 volume was observed for any of the phenolic acids at all time periods.

Composition of the microflora in the lumen and SCFA analysis. The mean composition of the microbiota did not differ for the 5 experiments. For *Bifidobacterium*, *Bacteroides*, *Clostridium,* E*nterococcus*, *Lactobacillus*, and *Enterobacteriaceae*, compositions at the beginnings of the 5 experiments were the same as at the ends (8.7 ± 1.3 to 12.5 ± 0.2 log colony forming units/L). The formation of SCFA was linear during the 28 h of incubation and there were no changes in microorganism composition between the beginning and the end of the fermentation (**Fig. 4**), which confirmed that there was no growth inhibitory effect of the supplements on the microorganisms.

Antiproliferative activity. The antiproliferative activity of all phenolic acids that occurred in the in vitro colon simulation was determined in LNCaP cells compared with parent flavonoid compounds representative of each flavonoid group (**Table 2**). 3,4DHPAA had the highest antiproliferative activity of the phenolic acids (IC_{50} = 135 μmol/L). Among the parent flavonoids, EGCG exhibited the highest activity (IC_{50} = 29 μmol/L). The antiproliferative activity of 4 phenolic acids, which showed antiproliferative activity in LNCaP cells, was also tested in HCT116 colon cancer cells (Table 2). Again, 3,4DHPAA was the only phenolic acid exhibiting considerable antiproliferative activity (IC_{50} = 90 μmol/L). The antiproliferative activity of 3,4DHPAA was further compared between a colon cancer (HCT116) and normal colon (IEC6) cell line (**Fig. 5**). 3,4DHPAA exhibited a significantly higher antiproliferative activity in the colon cancer cells compared with the normal colon cell line (Fig. 5).

DISCUSSION

The major phenolic acid formed in the present in vitro colon model study from all 4 sources of flavonoids was 3-methoxy-4-hydroxyphenylacetic acid also called homovanillic acid. Because none of the infused flavonoids except for hesperidin

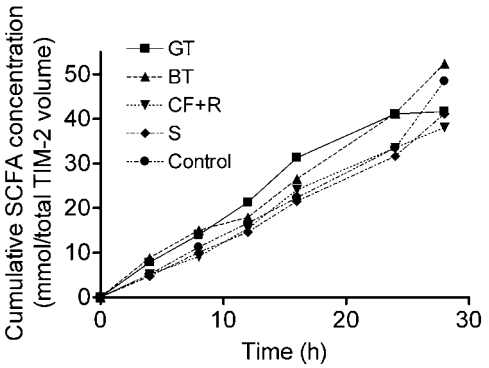

FIGURE 4 Cumulative SCFA production in lumen and dialysate after GT, BT, CF+R, and S infusion. The following SCFA were analyzed: acetate, propionate, butyrate, valerate, isobutyric acid, and isovalerate. Values are single measurements.

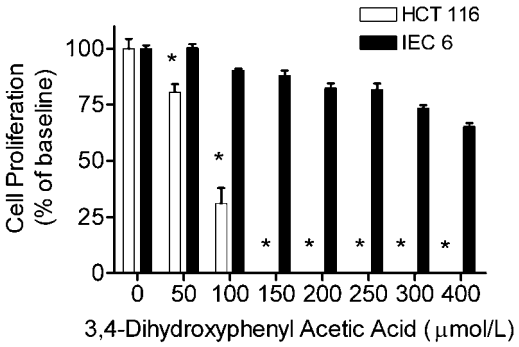

FIGURE 5 Growth inhibition of colon cancer cells (HCT116) and normal colon cells (IEC6) by 3,4DHPAA. Values are means ± SD, $n = 3$–4. *Different from HCT116, $P < 0.005$.

contained the oxomethyl group in the B-ring, the fermentation products must have undergone methylation during the fermentation process. 3M4HPAA was also found previously in an in vivo study by Rechner et al. (8) who demonstrated increased urinary and plasma levels of simple phenolic acids such as 3M4HPAA and 3HPAA after the ingestion of a flavonoid-rich meal. However, in another vivo study, Aura et al. (18) demonstrated that the main metabolite from rutin was 3,4DHPAA. In this study, no methylated hydroxyphenylacetic acids were found (18). Possible differences in products between fermentation studies are the origin and composition of the microbiota and the length of time of fermentation. In the present study, the composition of the pooled microbiota was identified and microorganisms were quantitated. This information will be beneficial for comparison with future studies.

Additional in vitro fermentation studies of naringin, rutin, and ECG using simpler in vitro models were published and found phenolic acids similar to those in this study. For example, Rechner et al. (19) found the following fermentation products: 3,4HPPA and 3-phenylpropionic acid from naringin and 3HPAA and 3,3HPPA from rutin after 48 h of fermentation. In the study by Meselhy et al. (6), EGC was fermented with human intestinal bacteria. They identified 15 fermentation products. However, no information was given for a quantitative comparison of the contribution of each product (6).

Compared with GT, BT undergoes fermentation during the manufacturing process, leading to the formation of larger polymers of tea polyphenol units called theaflavins and thearubigins (20). The BT infusion used in the present study contained 156 μmol EGC. The maximum cumulative amount of EGC determined in the lumen and dialysate was 350 μmol 16 h after the start of the experiment. EGCG decreased from 507 to 12 μmol. It is likely that EGC was formed from EGCG and from thearubigins of BT.

One of the main characteristics in the structure of the phenolic acid/fermentation products is the presence or absence of 2 adjacent hydroxyl groups in the B-ring (catechol group), which most likely are derived from the B-ring of the flavonoid structure. The evaluation of the percentage of phenolic acids formed containing the catechol structure was 66% from GT, 76% from BT, 80% from CF+R, and 45% from S flavonoids. In the tea experiments, the catechol groups possibly were derived from the B-ring gallate group of EGC and EGCG. In the CF+R group, hesperidin, eridictyol, and quercetin contain the catechol group in the B-ring, whereas for the soy flavonoids, both daidzein and genistein have only 1 hydroxyl group in the B-ring. However, only a relatively small amount of the fermentation products that were formed from genistein and daidzein remained in the lumen. In addition, the daidzein metabolite equol was found in small amounts, reaching a maximum at 16 h. It appears that equol was further converted to smaller phenolic acids because it was not found in the samples collected at 28 h.

In other studies investigating the total human metabolism of tea flavonoids in vivo, hippuric acid was a major urinary metabolite (21,22). In the present study, only a very small amount of hippuric acid was present in the lumen content after the fermentation of CF+R and none after the infusion of GT, BT, or S. Therefore, we concluded that flavonoids are degraded in the colon to hydroxyphenylacetic acids and hydroxyphenylpropionic acids, and absorbed into the circulation where they undergo further metabolism to hippuric acid. During this metabolism, hydroxyphenylacetic and hydroxyphenyl propionic acids are possibly degraded to benzoic acid, which in turn can be conjugated to hippuric acid in the liver and kidney and excreted into the urine (23).

In summary, comparing the quantity and type of phenolic acids produced after the infusion of the 4 different flavonoid sources demonstrated that the major phenolic acid formed from all 4 flavonoid sources was 3-methoxy-4-hydroxyphenylacetic acid. CF+R flavonoid infusion yielded the largest variety of fermentation products in the highest quantity. The S isoflavone genistein was fermented to yield 2 products and daidzein remained mainly intact. GT and BT fermentation produced an intermediate variety and quantity of phenolic acids compared with the other 2 flavonoid sources.

TABLE 2

Antiproliferative activities (IC_{50}) of flavonoids and phenolic acids determined in the LNCaP prostate cancer and HCT116 colon cancer cell lines[1]

	IC_{50} μmol/L		IC_{50} μmol/L	
Flavonoid	LNCaP	Phenolic acid	LNCaP	HCT116
Naringenin	>80	3M4HPAA	>200	>200
Genistein	50	4HPAA	>200	—[2]
EGC	36	3,4DHPAA	135	90
EC	>100	3,3HPAA	>200	>200
EGCG	29	2,4,6THBA	>200	—[2]
ECG	>100	3HPAA	>200	—[2]
Theaflavins	37	Hippuric acid	>200	>200

[1] There were 3–4 replicates for each concentration.
[2] Not determined for HCT116 cells.

Our knowledge of the antiproliferative activity of phenolic acids is limited, whereas the antiproliferative activity of parent flavonoid compounds has been studied more frequently (22,23). In the present study EGCG, EGC, and the theaflavins exhibited the strongest antiproliferative effect compared with genistein and naringenin as determined in LNCaP cells. A comparable IC_{50} for genistein in LNCaP cells was demonstrated by Ouchi et al. (24). Among the phenolic acid degradation products tested in the present study 3,4DHPAA was the only phenolic acid exhibiting a considerable antiproliferative effect in LNCaP prostate cancer and HCT116 colon cancer cells, which also was found by Kim et al. (22). The antiproliferative activity of 3,4DHPAA may be due to its catechol structure. A similar association of the catechol moiety in the B-ring with antiproliferative activity was demonstrated for flavanones (23). The mechanism of the antiproliferative activity of the phenolic acids has not yet been investigated. The mechanisms described for the antiproliferative activity of flavonoids include an increase in apoptosis as observed for tea polyphenols (25), alteration of the cell cycle such as a reduction of cdc2 and cyclin B1 proteins as observed for apigenin (26), p53-dependent Fas-mediated pathways (25), or inhibition of receptor tyrosin kinases and related downstream pathways of signal transduction (27). A possible difference between phenolic acid and parent flavonoids may result from a decreased uptake of phenolic acids into the cells. For naringenin and tea polyphenols, we were able to demonstrate that they are readily absorbed into cultured colon cells (28). However, in a preliminary study we were not able to detect any of the phenolic acid mixture in HCT116 cells (unpublished data).

We also demonstrated that normal colon cells were highly resistant to 3,4DHPAA compared with the colon cancer cells. A similar effect was described for EGCG (29). Ahmad et al. (29) found that EGCG caused inhibition of cell growth and induction of apoptosis in human epidermoid carcinoma cells, but not in normal cells. The authors suggested that this was due to EGCG inhibiting nuclear factor-κB activation only in carcinoma but not in normal cells as reviewed by Shimizu and Weinstein (27).

The question arises whether the IC_{50} concentration of 90–135 μmol/L is physiologically achievable. The intestinal lumen content of small aromatic/phenolic compounds showed a mean of 932 μmol/24 h and 66 (8–157) μmol of dihydroxy aromatic/phenolic compounds in fecal water after the consumption of 3.4 daily servings of fruits and vegetables (30). Therefore, we concluded that the concentration of the phenolic acid products formed from flavonoids is high enough to contribute to the antiproliferative effect in the colon.

In summary, we demonstrated that an in vitro model (TIM-2), which mimics the microbiotic environment of the colon, can be used to study fermentation processes in the colon and to compare the fermentation products from different parent compounds under identical conditions. 3,4-dihydroxyphenylacetic acid, one of the phenolic acid fermentation products, has promising biological activity against cancer cell proliferation in vitro and warrants further investigation.

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