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Gut Microbial Metabolism of Polyphenols from Black Tea and Red Wine/Grape Juice Is Source-Specific and Colon-Region Dependent

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Supporting Information

ABSTRACT: The colonic microbial degradation of a polyphenol-rich black tea extract (BTE) and red wine/grape juice extract (RWGE) was compared in a five-stage in vitro gastrointestinal model (TWINSHIME). Microbial metabolism of BTE and RWGE polyphenols in the TWINSHIME was studied subsequently in single- and continuous-dose experiments. A combination of liquid or gas chromatography with mass spectrometry (LC-MS or GC-MS) and NMR-based metabolic profiling was used to measure selected parent polyphenols, their microbial degradation into phenolic acids, and the production of short-chain fatty acids (SCFAs) in different colon compartments. Acetate production was increased by continuous feeding of BTE but not RWGE. During RWGE feeding, gallic acid and 4-hydroxyphenylpropionic acid remained elevated throughout the colon, while during BTE feeding, they were consumed in the distal colon, while 3-phenylpropionic acid was strongly produced. Gut microbial production of phenolics and SCFAs is dependent on colon location and polyphenol source, which may influence potential health benefits.

KEYWORDS: *Flavonoids, catechins, phenolic acids, gut microbiota, metabolism, metabolomics*

INTRODUCTION

Epidemiological studies have shown that dietary intake of polyphenols is associated with health benefits in humans, particularly with risk reduction of cardiovascular diseases and cancer. However, intervention studies show more heterogeneous effects on cardiovascular disease risk markers for different polyphenol sources.[1] Polyphenols are abundantly present in many fruits and vegetables, chocolate, tea, and red wine and constitute a wide variety of phenolic compounds. Black tea contains predominantly oligomeric polyphenol compounds called thearubigens and also theaflavins, catechins, and phenolic acids. Red wine and grapes mainly contain proanthocyanidins, as well as anthocyanins, catechins, and phenolic acids. Bioavailability of the oligomeric polyphenols (e.g., proanthocyanidins and thearubigens) and of the intact monomeric polyphenols (e.g., catechins and anthocyanins) is generally poor. Only a small fraction of polyphenols is absorbed from the small intestine as aglycons after enzymatic deglycosylation.[2] A major fraction of dietary polyphenols reaches the colon intact and is subsequently degraded by gut microbiota into simpler phenolic acids, which can be absorbed into the systemic circulation.[3,4] Extensive gut microbial fermentation has been reported for polyphenols from wine [5] and black tea,[6] and since phenolic catabolites may achieve higher levels than the intact polyphenols, they potentially contribute importantly to local or systemic health benefits. The microbial fermentation of various polyphenol classes has been studied, showing that many of the phenolic acids and lactones produced seem to be rather generic and not only linked to a single parent compound.[7,8] Polyphenol-rich dietary interventions in humans have demonstrated high interindividual variability in concentrations of phenolic acids excreted in urine, which may be linked to microbial populations present in the gut and their activity.[9,10] High interindividual variability in polyphenol degradation patterns was also observed in an in vitro batch fermentation model using fecal samples from different human volunteers.[11] The interaction between gut microbiota and polyphenols is actually mutual, since prolonged exposure to dietary polyphenols may also directly affect gut microbial population and/or activity.[8,12−14] The effects of polyphenol feeding on bacterial growth in in vitro gut fermentation models varied for different bacterial strains and included potential bifidogenic effects, suggesting that polyphenol-rich diets could support gut health through their prebiotic activity. Although in vitro gut models cannot completely mimic the in vivo human digestive system, they have some advantages over in vivo studies. First, in vitro models allow focusing specifically on gut microbial activity and thus reduce some of the complexity caused by interactions with the host, for example, at the level of the gut epithelium. Second, microbial population and microbe-mediated metabolic effects can be dynamically monitored as a function of time and even in

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different parts of the colon, as in the simulator of the human intestinal ecosystem (SHIME) model.[15,16] In human studies, monitoring of dynamic processes inside the gut is impossible, due to limited opportunities to collect intestinal samples. Dynamic multireactor gastrointestinal models like SHIME offer better simulation of complex conditions present in the human gut than simple in vitro batch fermentations.

The aim of this study was to compare the gut microbial catabolism of a polyphenol-rich black tea extract (BTE) and a mix of red wine/grape juice extracts (RWGE) in different compartments of the colon in vitro. The effects of a single dose of black tea or RWGE were compared to long-term effects of 2-week continuous dosing. In this study, a TWINSHIME system was used, consisting of two five-compartment in vitro gastrointestinal models running in parallel, both inoculated with the same faecal sample to provide equal starting microbiota composition for both treatments. Liquid or gas chromatography with mass spectrometry (LC-MS or GC-MS) and NMR-based metabolic profiling were used to monitor selected precursor polyphenols and a variety of microbially produced phenolic acids and short-chain fatty acids.

MATERIALS AND METHODS

Ingredients. Two different polyphenol sources, a mixture of red wine and grape juice extract (RWGE) and a black tea extract (BTE), were studied. RWGE was composed of two parts of Provinols red wine extract (Seppic, France) and one part of MegaNatural Rubired grape juice extract (Polyphenolics, USA). BTE was prepared by spray-drying from Lipton Yellow Label (LYL) black tea (Unilever, The Netherlands). RWGE and BTE contained 57% and 32% total polyphenols, respectively. The RWGE composition is described in more detail in a recent paper from our laboratory.[17] The polyphenol content of LYL black tea has been described by Mulder et al.[18]Table S1 (Supplementary Information) summarizes the known phenolic composition of BTE and RWGE.

TWINSHIME Experiment. SHIME (Ghent University-ProDigest, Ghent, Belgium) consists of five successive reactors, simulating the stomach, small intestine, colon ascendens (CA), colon transversum (CT), and colon descendens (CD).[15] The microbial community in the last three reactors is derived from a fresh human fecal sample. Feeding medium and pancreatic fluid were added to the system at regular intervals. Automatic control of temperature, pH, and retention time aimed to ensure the development and maintenance of a microbial community that corresponds to in vivo conditions in the human colon. In this study, a TWINSHIME setup was used by operating two systems in parallel, with reactor setup, inoculum preparation, and reactor feed composition essentially as described by Possemiers et al.[16] Reactor feed consisted of the following components: 1 g/L arabinogalactan, 2 g/L pectin, 1 g/L xylan, 3 g/L potato starch, 0.4 g/L glucose, 3 g/L yeast extract, 1 g/L peptone,4 g/L mucin, and 0.5 g/L cysteine. The pH of the feed was set to 2, and the feed was stored at 4 °C before administration to reactor 1. Both systems were operated under identical pH and temperature conditions, and all colon vessels were inoculated with the same fecal sample from a healthy human volunteer. The TWINSHIME setup allowed for parallel investigation of microbial bioconversion of the two polyphenol-rich extracts, BTE and RWGE. BTE was administered to one system and RWGE to the other system. After inoculation, the TWINSHIME systems were first allowed 3.5 weeks to reach steady state. Baseline samples were collected during one control week. After that, a single dose of the polyphenol extracts (1 g of polyphenols) was applied to the stomach compartments of both reactor systems. Starting 1 week later, the polyphenol extracts were administered to the system three times daily (1 g of polyphenols/day) by adding the extracts to the regular SHIME feeding medium for 2 weeks. Finally, a 2-week washout period without polyphenol dosing was included at the end of the experiment. The polyphenol doses were selected to simulate physiological conditions since the total dietary polyphenol intake for humans has been estimated to be approximately 1 g/day or even considerably higher in different countries.[19,20] The moderately high dose in the current study was chosen to ensure detection of the polyphenolic compounds and their breakdown products by NMR and/or GC-MS when background signals from the complex SHIME medium were considered. During the whole study, samples were collected at regular time points from the three colon vessels and stored at −20 °C until further analysis.

^{1}H NMR Profiling. Levels of short-chain fatty acids (SCFAs) and caffeine were assessed by ^{1}H NMR spectroscopy, essentially as described previously.[11] In brief, SHIME medium samples were defrosted and then centrifuged (5 min, 21912*g*, 6 °C). NMR samples were prepared by taking 450 μL of the SHIME medium supernatant and adding 50 μL of deuterium oxide (D_2O) containing 10 mM sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d_4 (TSP-d_4). High-resolution one-dimensional (1D) ^{1}H NMR spectra were acquired on a Bruker Avance 600 NMR spectrometer operating at 600.13 MHz and at a temperature of 300 K. A 5 mm TXI probe and a sample changer for sample delivery were used. A standard 1D water-suppressed pulse sequence was used (noesygppr1d, Bruker Biospin, Germany); 64 scans were acquired with 32k data points over 8993 Hz, with a relaxation delay of 3 s and a mixing time of 150 ms. The spectra were manually corrected for phase and baseline distortions using Topspin 1.3 software (Bruker Analytik, Rheinstetten, Germany). Metabolites were identified where possible by use of a reference NMR spectral database (bbiorefcode-2-0-0 implemented in Amix 3.8.3, Bruker Biospin GmbH). Metabolite quantification was performed by peak integration and normalization to the known concentration of the internal standard, TSP-d_4, by use of the Amix software.

Targeted GC-MS Profiling. The production of a wide range of phenolic acids was quantified by GC-MS, as described previously.[11,21] Briefly, 0.4 mL aliquots of the SHIME sample were centrifuged, mixed with the internal standard *trans*-cinnamic acid-d_6 (Sigma−Aldrich, St. Louis, MO), acidified, and then extracted three times with ethylacetate. The dried extracts were derivatized with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and 10% trimethylchlorosilane (TMCS) and mixed with *n*-hexane prior to GC-MS analysis. A quadrupole-MS analyzer (Agilent MSD) interfaced with an Agilent 7890A gas chromatograph was used. Full scanning mass spectrometry was applied for all analysis in the electron-ionization (EI) mode at 70 eV with the ion source maintained at 180 °C. Calibration standards were prepared from a phenolic acid stock solution containing a mix of 37 different phenolic acids at a concentration of 1 mg/mL (see Table S2, Supporting Information).[11] Eleven-point calibration lines were prepared for quantification by serial dilution of the phenolic acid standard mix.

LC-MS Analysis. For the LC-MS target analysis of precursor polyphenols and selected phenylvalerolactones, 0.5 mL of the defrosted SHIME samples was acidified with 10 μL

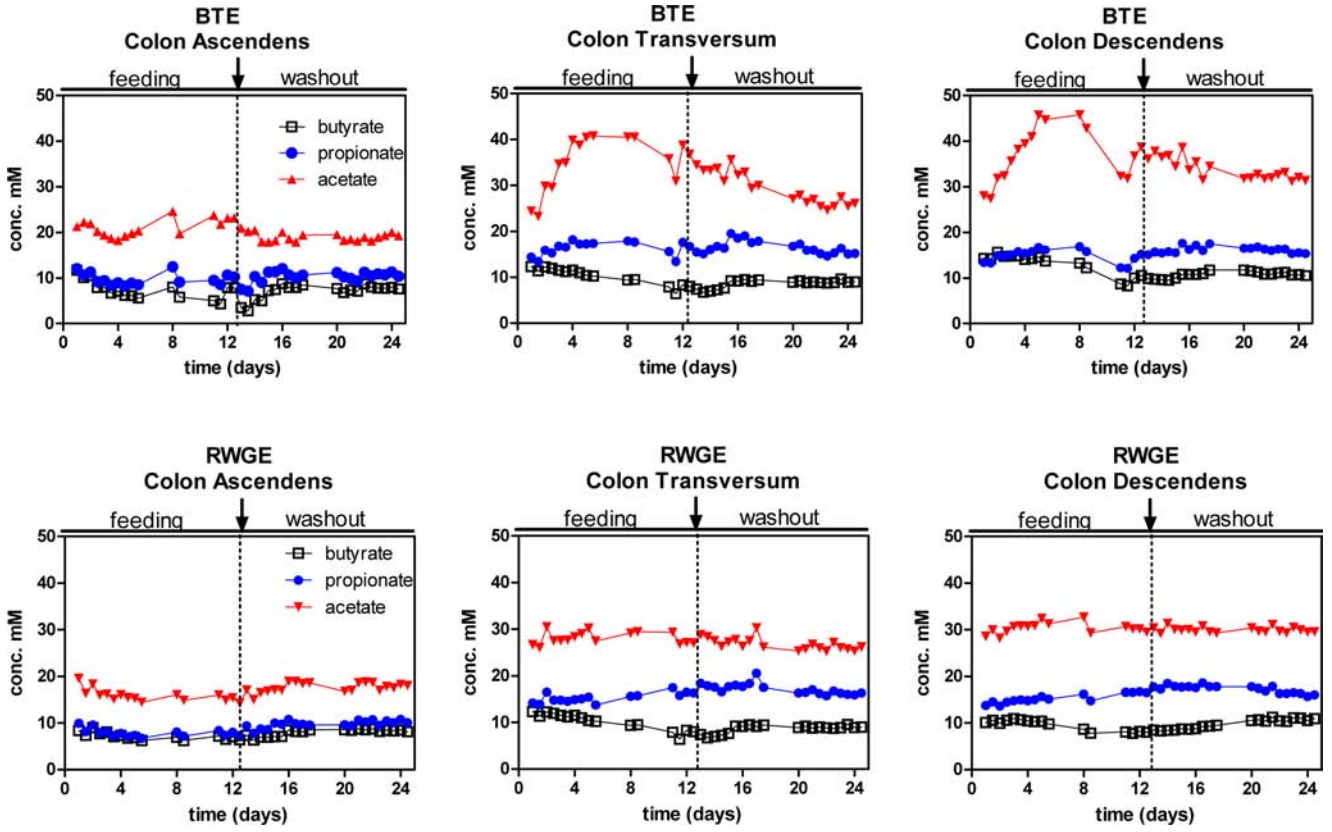


Figure 1. Temporal changes in levels of SCFAs acetate (solid red triangles), propionate (solid blue circles), and butyrate (open black squares) during 2-week continuous dosing of BTE (top row) or RWGE polyphenols (bottom row) and during the subsequent 2-week washout period. SCFA levels (millimolar) in the CA, CT, and CD compartments are shown from left to right.

(25.5%) of formic acid (FA). After 15 min the samples were sonicated for 10 min, centrifuged for 10 min at 2500 rpm, filtered through a 0.45 μm inorganic membrane filter, and finally transferred into an HPLC vial. The LC-MS system consisted of an Accela HPLC system with an Accela photodiode array (PDA) detector, connected to a LTQ/Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) that was equipped with an ESI source, controlled by Xcalibur 2.1 software. Sample injection volume was 5 μL and liquid chromatography separation was achieved on a Luna C18(2) analytical column (150 × 2 mm, 3 μm particle size, Phenomenex, Torrance, CA). Mobile phase A consisted of water with 0.1% (v/v) FA, and mobile phase B was acetonitrile (ACN) with 0.1% (v/v) FA. A solvent gradient was applied starting from 5% B and increasing to 35% B in 45 min. In addition, the column was washed and equilibrated for 15 min before the next sample injection. The flow rate was 0.19 mL/min, and column and autosampler temperatures were set at 40 and 4 °C, respectively. The total run time was 60 min. Orbitrap FT-MS settings were as described recently.[22] In short, masses in the range m/z 100−1500 were recorded with a resolution of 60.000 (at m/z 400), and with a full automatic gain control (AGC) target of 200 000 charges, by use of two microscans. The capillary temperature was 275 °C, sheath gas flow was 60 au, auxiliary gas flow was 5 au, and sweep gas flow was 5 au. The Orbitrap was externally calibrated in negative mode by use of sodium formate clusters in the range m/z 150−1200, and automatic tuning was performed on m/z 384.93. The Ion Trap was set at an AGC target value of 30 000. Quantification of target polyphenols was performed by using 7-point calibration curves obtained with commercially available polyphenol standards (Sigma−Aldrich, St. Louis, MO). The phenyl-valerolactone standards used for quantification, (−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (3,4-dHPVL) and (−)-5-(3′-methoxy-4′-hydroxyphenyl)-γ-valerolactone (3M4HPVL), were obtained by chemical synthesis and subsequent purification (Unilever Research China).

RESULTS

Polyphenol Treatment Affects Microbial Short-Chain Fatty Acids. ^{1}H NMR metabolic profiling was used to quantify levels of short-chain fatty acids (SCFAs) in samples from the SHIME colon vessels (CA, CT, and CD) at regular time points. After a single BTE dose administration, there was a slight increase in production of acetate and propionate, predominantly in the transverse colon (CT) during the first 48 h (Figure S2, Supporting Information). This was not the case after a single RWGE dose. Butyrate levels did not markedly change after BTE or RWGE dosing. As shown in Figure 1, acetate production increased in CT and CD during continuous dosing of BTE but not during continuous dosing of RWGE. During both BTE and RWGE feeding, a slight increase in propionate production was observed in CT and CD, along with a reduction in butyrate production in these compartments.

Caffeine as a Model for SHIME Transport Dynamics. After dosing of BTE, caffeine was identified in samples of the three SHIME colon vessels (CA, CT, and CD) by ^{1}H NMR profiling. The concentration−time profiles of caffeine are shown in Figure 2. Although there is some literature that caffeine may be degraded by certain environmental bacteria like

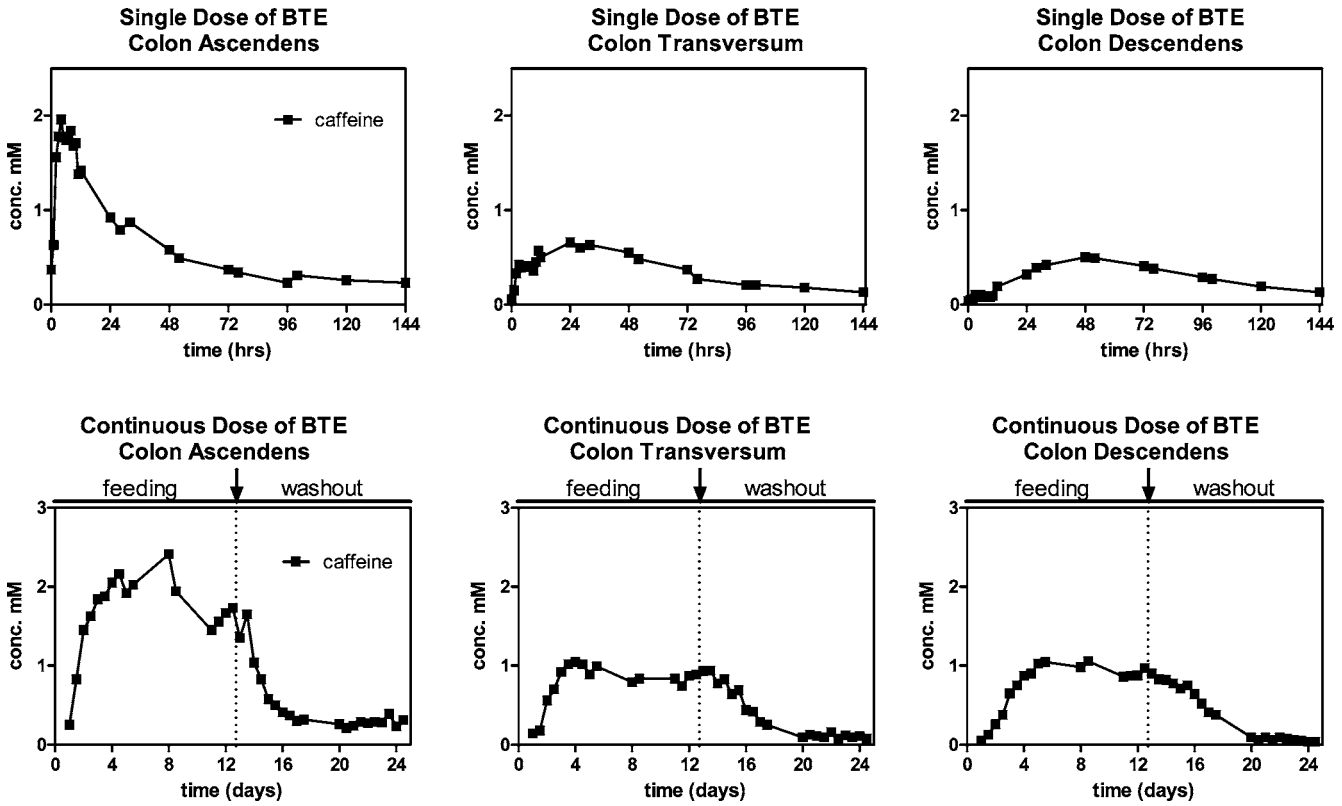


Figure 2. Caffeine kinetics used as a reference model for the single-dose SHIME experiment and for continuous feeding experiments. (Top row) After a single dose of BTE, the kinetics of caffeine, which is considered an "inert" reference compound, was monitored to model the SHIME transport dynamics. (Bottom row) During continuous feeding of BTE at 1 g/day, caffeine levels in the SHIME vessels reach a steady state after approximately 4 days, and during the washout period, caffeine levels return to baseline.

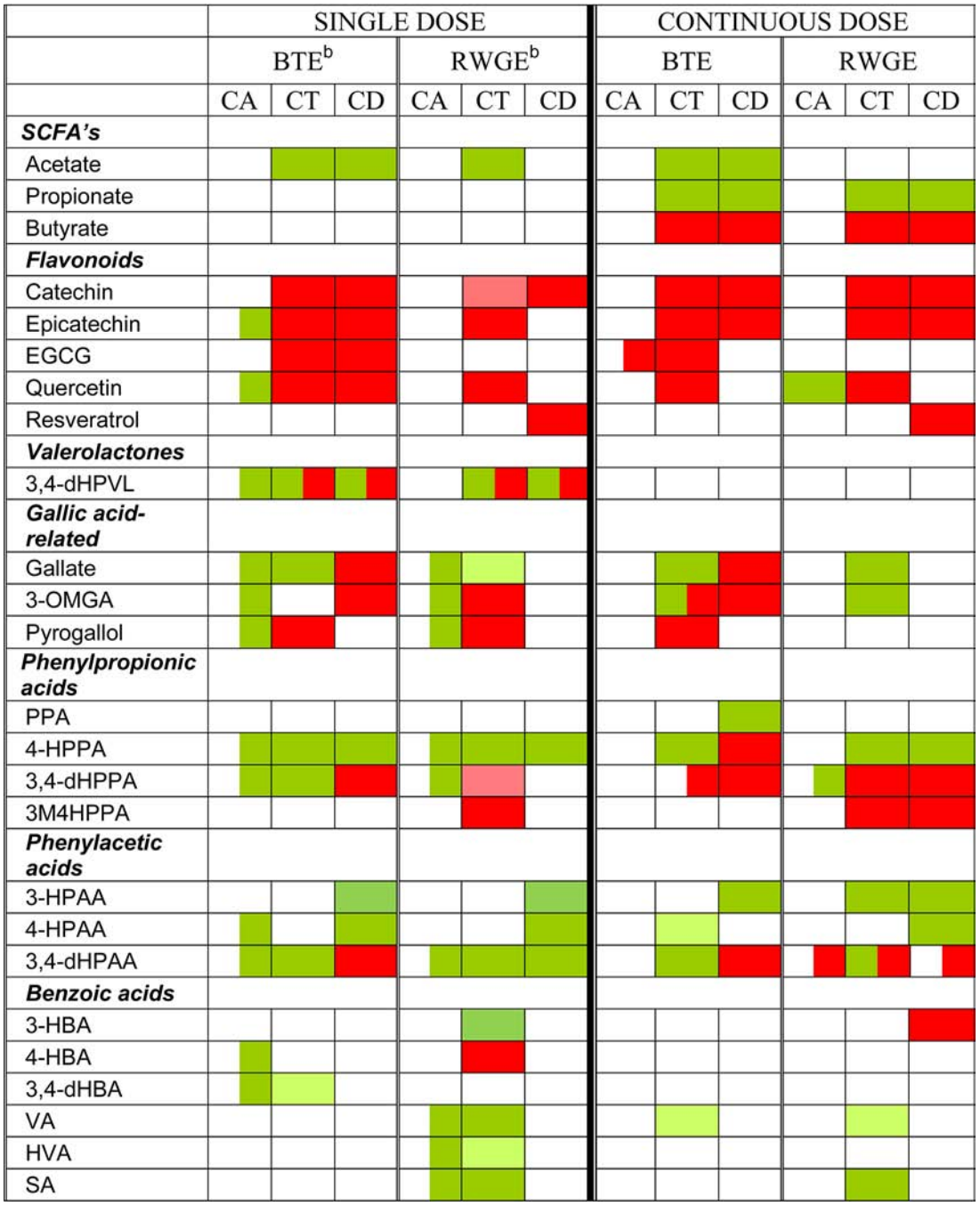
Pseudomonas putida,[64] caffeine is largely resistant to degradation by human gut bacterial enzymes, as is also shown in Figure 2. It was therefore used as a reference compound to model the transport dynamics of the SHIME system. After single-dose BTE administration, the kinetic profile of caffeine showed a rapid increase, peaking at 4−8 h in the CA compartment with a slower decay up to t = 144 h. Peak concentrations in the subsequent CT and CD compartments appeared later, at around 24 and 48 h, respectively, with the overall damped shape of the kinetic curve reflecting the gradual transport of fluid and caffeine from CA via CT to CD vessels. During continuous feeding of BTE, the caffeine level in the CA compartment rose steadily until it reached a plateau level at around 4 days, when the influx and efflux of caffeine in a given compartment/vessel were at equilibrium. When polyphenol feeding was stopped after 2 weeks, the caffeine content in CA returned to baseline within 4−6 days. Similar patterns were observed for CT and CD compartments. Plateau levels of caffeine were also reached after about 4 days, although they were lower, reflecting the gradual dilution of caffeine with transport from CA via CT to CD vessel.

Kinetics of Polyphenols and Catabolites after a Single Dose of BTE or RWGE. Concentration−time curves for the identified polyphenols and phenolic acids for each of the colon vessels are depicted in Figures 3 and 4. A qualitative comparison of the shape of the time course profiles of individual metabolites in subsequent colon vessels was made and contrasted against the time course of caffeine (Figure 2). This was used to infer microbial polyphenol consumption/degradation, which was characterized by a relatively strong reduction in peak concentrations with respect to the preceding vessel and when compared to the caffeine time course. Conversely, increased polyphenol metabolite production was indicated by a relative increase in peak concentrations with respect to the preceding vessel and when compared to the caffeine time course. Table 1 summarizes the overall changes in SCFAs, intact polyphenols, and polyphenol catabolites as observed in the TWINSHIME model after single-dose BTE and RWGE.

Concentration−time curves for (−)-epicatechin, (+)-catechin, EGCG, and resveratrol after single-dose administration are depicted in Figure 3. Levels of the catechins increased rapidly in the CA vessel, reaching a maximum 4−8 h after the single dose of BTE or RWGE, which can be explained by their presence in the extracts. EGCG was primarily present after BTE dosing. A second peak occurring in CA 24−36 h after dosing, suggested some additional production of catechin and epicatechin. The catechins were further degraded in CT and CD compartments. Quercetin also appeared initially in CA after BTE dosing and to a lesser extent after RWGE (see Figure S3, Supporting Information). Since quercetin was no longer detected in CA after 24 h and was not detected in CT and CD compartments at any time point, it was apparently rapidly degraded. Resveratrol, which is a component of RWGE, appeared in CA and CT and was fully converted in CD.

One of the initial microbial ring-fission catabolites of catechins and procyanidins, (−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (3,4-dHPVL),[7,23−26] was quantified by LC-MS (see Figure S4, Supporting Information). After single-dose administration of BTE, 3,4-dHPVL was detected in CA but

Table 1. Overview of Observed Changes in Microbial Metabolite Production in the SHIME Model Following Single or Continuous Dose of BTE or RWGE Polyphenols[a]



[a]Colors in each cell denote qualitative increase (green) or decrease (red) in phenolic production in the corresponding colon compartment (CA, CT, or CD), when the response in the preceding vessel is considered and the general transport kinetics, as observed for caffeine, are taken into account. Light green and red indicate a weak increase or decrease, respectively. Half-colored cells indicate early response (left half colored) or late/secondary response (right half colored). [b] The early peak of phenolics, as observed in the CA compartment after a single dose of tea/RWGE and suggesting the presence of this compound in the extract itself, is *not* shown here.

reached highest levels in the CT vessel starting around 6−8 h and peaking at 32 h postdose. Decreasing levels after 48 h indicated further degradation, particularly in CT and CD compartments. After the single dose of RWGE, 3,4-dHPVL was not observed in CA, but it was produced in CT and CD to higher levels than after BTE polyphenol dosing. The related compound (−)-5-(3′-methoxy-4′-hydroxyphenyl)-γ-valerolactone (3M4HPVL) was not detected in any of the experiments.

Gallic acid levels in the CA vessel increased rapidly (Figure 3), within 3−4 h after BTE or RWGE dosing, which is consistent with its presence in both extracts. Between 24 and 48 h after dosing, some additional production of gallic acid was observed in CA and CT, while consumption was observed in CD. Also, potential gallic acid metabolites 3- and 4-*O*-methylgallic acid (3-OMGA and 4-OMGA) were observed after BTE and RWGE dosing, albeit at much lower levels than

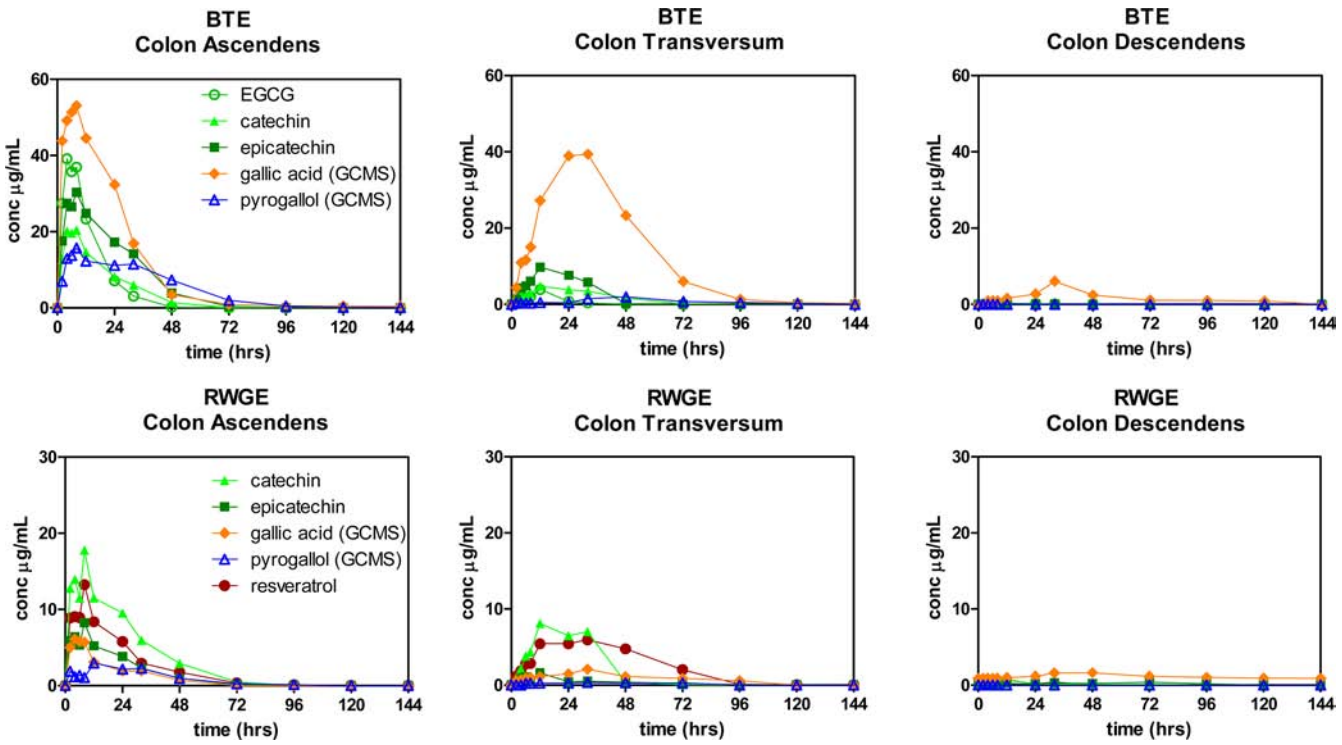


Figure 3. Kinetics of main parent polyphenols and valerolactone in different SHIME colon compartments following a single dose (1 g) of BTE (top row) or RWGE (bottom row) polyphenols. Colon vessels 3−5 of the SHIME model are shown from left to right. The depicted polyphenols are EGCG (open green circles), (−)-epicatechin (solid green squares), (+)-catechin (solid green triangles), gallic acid (solid orange diamonds), pyrogallol (open blue triangles), and resveratrol (solid red circles).

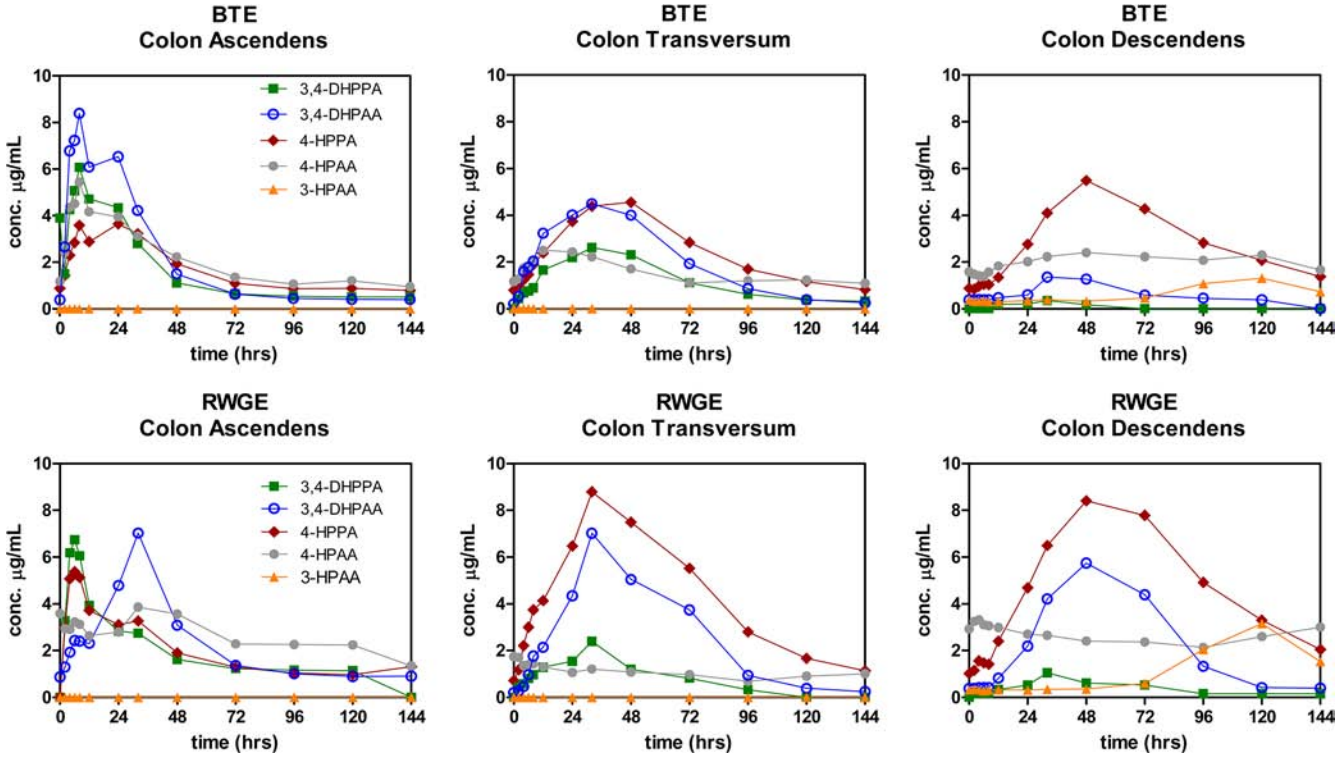


Figure 4. Kinetics of microbial phenolic acids produced in the different SHIME colon compartments following a single dose (1 g) of BTE (top row) or RWGE (bottom row) polyphenols. Colon vessels 3−5 of the SHIME model are shown from left to right. Symbols for the depicted phenolic acids are as follows: 3,4-dHPPA (solid green squares), 4-HPPA (solid red diamonds), 3,4-dHPAA (open blue circles), 4-HPAA (solid gray circles), and 3-HPAA (solid orange triangles).

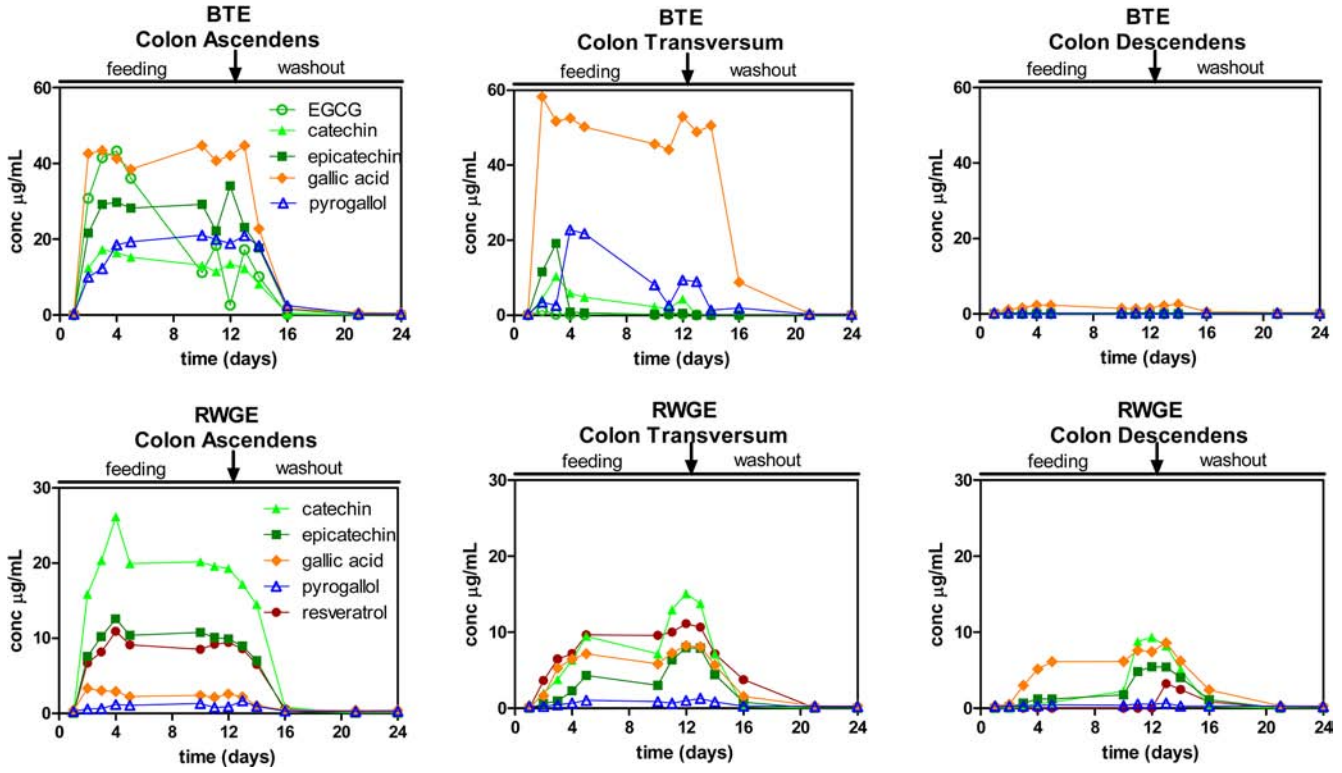


Figure 5. Kinetics of main parent polyphenols and valerolactones in the different SHIME colon compartments following continuous dosing of BTE (top row) or RWGE (bottom row) polyphenols (1 g/day). Colon vessels 3−5 of the SHIME model are shown from left to right. Symbols for the depicted polyphenols are as follows: EGCG (open green circles), (−)-epicatechin (solid green squares), (+)-catechin (solid green triangles), gallic acid (solid orange diamonds), pyrogallol (open blue triangles), and resveratrol (solid red circles).

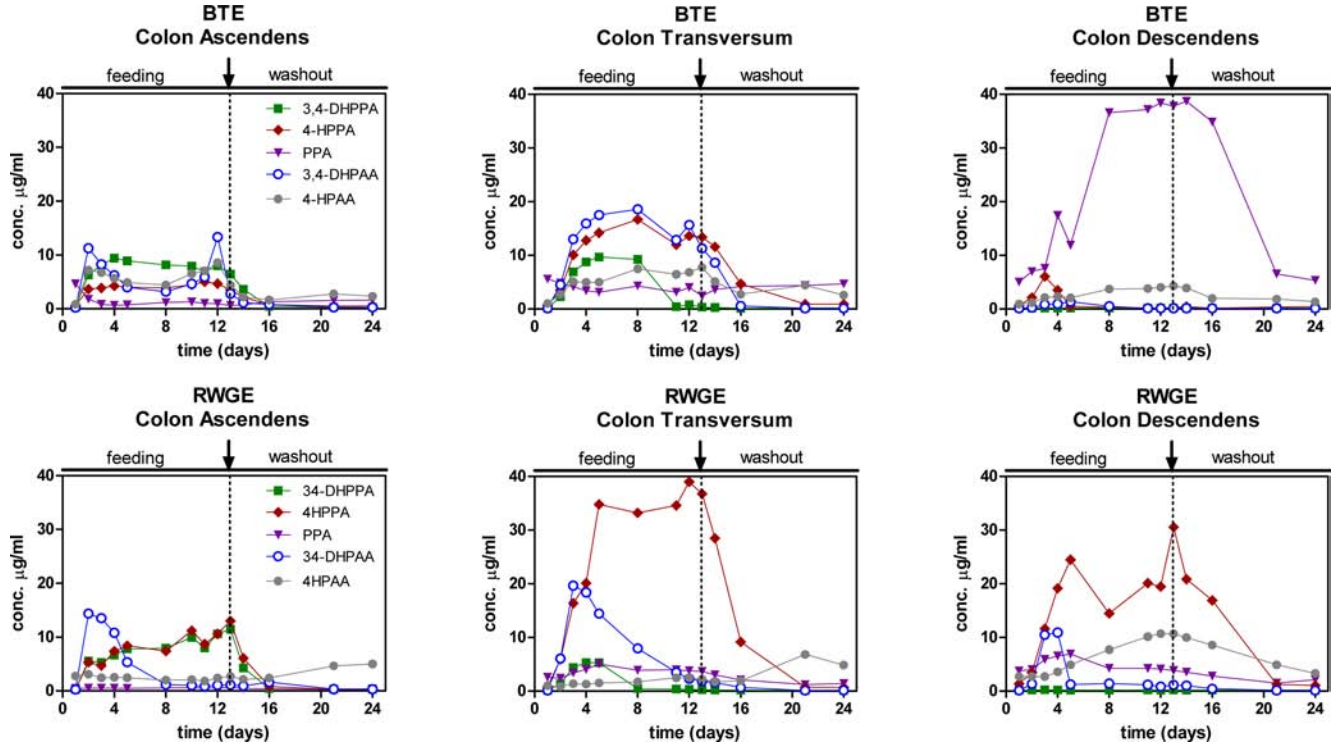


Figure 6. Kinetics of microbial phenolic acids produced in the different SHIME colon compartments following continuous dosing of BTE (top row) or RWGE (bottom row) polyphenols (1 g/day). Colon vessels 3−5 of the SHIME model are shown from left to right. Symbols for the depicted phenolic acids are as follows: 3,4-dHPPA (solid green squares), 4-HPAA (solid red diamonds), PPA (solid purple inverted triangles), 3,4-dHPAA (open blue circles), 4-HPAA (solid gray circles), and 3-HPAA (solid orange triangles).

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gallic acid (Figure S5, Supporting Information). Notable production of 4-OMGA was observed in CT and CD following RWGE dosing, while after BTE dosing predominantly 3-OMGA was produced in CA and CT. An increase in production of pyrogallol in CA was observed after BTE dosing, with the highest levels found 8 h after administration and with a secondary peak at 32 h (Figure 3). Pyrogallol was strongly reduced in the CT and CD vessels, indicating further conversion. After a single RWGE dose, pyrogallol production was much lower but showed a similar pattern.

Following the single BTE dose, production of 3,4-dihydroxyphenylpropionate (3,4-dHPPA), 4-hydroxyphenylpropionate (4-HPPA), 3,4-dihydroxyphenylacetate (3,4-dHPAA), and 3,4-dihydroxybenzoic acid was observed in CA and CT vessels (Figure 4). Subsequently, in the CD vessel, 3,4-dHPPA and 3,4-dHPAA were consumed or degraded, as indicated by markedly reduced levels versus those in CT, while production of 4-HPPA and 4-HPAA was observed.

Following the single dose of RWGE, the CA compartment showed some additional production of 3,4-dHPPA, 4-HPPA, and 3,4-dHPAA around 32 h (Figure 4). In the CT and CD compartments, markedly strong production of 4-HPPA was observed, while 3,4-dHPPA and 3-methoxy-4-hydroxyphenylpropionate (3M4HPPA) were degraded, particularly in CT. Minor increases in levels of 3-hydroxybenzoic acid (3-HBA), vanillic acid (VA), homovanillic acid (HVA), and syringic acid (SA) were observed in the CT vessel after a single RWGE dose, while 3-HBA production was also elevated in CD (Table 1; Figure S6, Supporting Information).

Metabolite Kinetics during Continuous Polyphenol Feeding and Washout. Concentration−time curves for the various polyphenols and phenolic acids in each colon vessel during continuous dosing are depicted in Figures 5 and 6, and overall effects are summarized in Table 1. During continuous feeding of BTE polyphenols, levels of (+)-catechin, (−)-epicatechin, and EGCG in CA reached a maximum around day 3 or 4 and remained more or less stable in this compartment until day 12, followed by a return to baseline during the washout phase (Figure 5). In CT and CD compartments these catechins were strongly reduced after 3−4 days, indicating an increased catechin degradation capability, possibly by changes in microbial population or activity. Resveratrol was produced in the CA and CT vessel during continuous RWGE feeding but was no longer detected in the CD vessel, suggesting that it was metabolized. During continuous BTE dosing, 3,4-dHPVL was detected in all vessels, while during continuous RWGE dosing production was not detected in the CA vessel. In CT and CD compartments, quercetin was fully degraded (see Figure S2, Supporting Information), and 3,4-dHPVL started to decrease from day 4 (see Figure S4, Supporting Information), suggesting an increase in microbial degradation activity.

During continuous feeding of BTE and RWGE polyphenols, a clear increase in gallic acid production was found in CT, as compared to the CA compartment. Remarkably, gallic acid production was still increased in the CD compartment during RWGE feeding, but production was very low in CD during BTE feeding (Figure 5). The production of 3-OMGA and 4-OMGA showed a similar pattern to that of gallic acid, but levels of the O-methylated metabolites were much lower (Figure S5, Supporting Information). During continuous BTE feeding, pyrogallol was more reduced in CT and CD compartments as compared to CA, indicating a stronger tendency for consumption of pyrogallol, whereas pyrogallol production was stable after RWGE feeding. During continuous feeding of BTE, an increase in production of 3,4-dHPAA and 4-HPPA was observed in CT (Figure 6). Subsequently, marked degradation of 3,4-dHPPA and 4-HPPA as well as 3,4-dHPAA was observed in the CD vessel, while there was a strong increase in production of 3-phenylpropionic acid (PPA) and a lesser increase in 3-HPAA. During continuous feeding of the RWGE polyphenols, marked degradation of 3,4-dHPPA was observed in both the CT and CD vessels (Figure 6). 3,4-dHPAA was degraded in all three colon compartments after approximately 4 days of feeding. Simultaneously, a strong increase in production of 4-HPPA (CT) and moderate increases in production of 3-HPAA (CT and CD) and 4-HPAA (CD) were observed. Also, syringic acid production increased markedly in CT while production of 3-HBA was reduced in CD (Figure S7, Supporting Information).

Changes in production of phenolic acids during continuous feeding of polyphenol-rich BTE and RWGE were generally reversible during the washout period, when polyphenol feeding was stopped (Figure 6).

DISCUSSION

This study explored the gut microbial degradation of dietary polyphenols in a five-stage gastrointestinal model system (TWINSHIME). In the single-dose experiment with BTE and RWGE polyphenols, the kinetics of microbial polyphenol degradation during transit through the simulated colon compartments was studied. The subsequent 2-week continuous-dose experiment aimed to study longer-term changes in metabolic activity, which may reflect adaptation of the colonic microbial composition to polyphenol feeding. Gut microbial fermentation of RWGE and BTE, two polyphenol-rich extracts with different but similarly complex polyphenol compositions, was found to produce different profiles of polyphenols and phenolic acids during transit through the model colon.

BTE and RWGE Modulate Microbial Short-Chain Fatty Acid Production. Changes in the microbial production of SCFAs after feeding BTE or RWGE polyphenols were most pronounced during the 2-week continuous feeding period. SCFAs may originate from the colonic microbial fermentation of carbohydrates present in the culture medium but possibly also from microbial conversion of polyphenols.[27] The major SCFAs—butyrate, acetate, and propionate— have been associated with potential health benefits extending beyond the gut epithelium.[28−30] Continuous feeding of BTE polyphenols caused a clear increase in acetate production in the CT and CD compartments of about 70%, while acetate production was not affected during continuous RWGE feeding. Both BTE and RWGE feeding induced a modest increase in propionate production in CT and CD and a slight reduction in butyrate. Changes in (relative levels of) individual SCFAs can induce protective effects in vivo,[30] but more research is needed in this area.

BTE and RWGE Polyphenols Undergo Different Microbial Catabolism. Although the colonic microbial fermentation products of various polyphenol-rich foods may share many commonalities, a few may be characteristic of a specific food source or polyphenol class.[7,31−34] The BTE and RWGE polyphenols and microbial metabolites identified here are in line with previous in vitro and human studies.

Catechins were present in both BTE and RWGE and they are initially degraded by colonic bacteria into phenyl-γ-valerolactones by ring fission of the C-ring, followed by

degradation into 3,4-dHPPA and subsequently into monohydroxylated phenylpropionic acids.[7,24−26,35] Also dimeric procyanidins are fermented mainly into 3,4-dHPVL, 3,4-diHPAA, and other unique catabolites[25,36,37] rather than into their constituent epicatechin monomers. In this study, the production of 3,4-dHPVL, the primary microbial metabolite of catechins, started already in CA after a BTE single dose, whereas after RWGE dosing it started only in CT (Figure S4, Supporting Information). After 32−48 h, 3,4-dHPVL was further converted in CT and CD. In this period, highest levels of hydroxyphenylpropionic acids and hydroxyphenylacetic acids, potential downstream metabolites of procyanidin dimers and monomeric catechins, were also found[7,36,38] (Figure 4). BTE and RWGE contained low levels of flavonols, such as quercetin, which are fermented in the colon into hydroxyphenylacetic acids, like 3,4-dHPAA, 4HPAA, and 3HPAA, and into phloroglucinol.[7,39,40] RWGE feeding also showed production of typical (microbial) metabolites of RWGE anthocyanins, such as protocatechuic acid (3,4-diHBA), syringic acid (SA), and/or vanillic acid (VA),[7,41] Particularly, the increased production of syringic acid in CT during continuous RWGE feeding may indicate adaptation of the microbiota to its likely precursor malvidin.[7,41] Resveratrol, an exclusive component of red wine, was only present at modest levels in CA and CT after RWGE dosing, and levels were markedly reduced in CD, indicating further conversion (Figure 4). Dihydroresveratrol has been suggested as a metabolite of resveratrol produced by the gut microbiota,[42,43] but further research in this area is needed.

In the single-dose experiment, both BTE and RWGE polyphenols were degraded into a variety of hydroxylated phenylpropionic acids, phenylacetic acids, and benzoic acids. In CA, differences in the early peak in phenolic acid levels between 4 and 8 h (Table 1) probably reflect the different phenolic acid content of BTE and RWGE. A second peak at 24 h may suggest additional production by microbial degradation of larger polyphenols. After a single dose of BTE, higher levels of GA and 4-HPAA were observed in the CA vessel, whereas after the RWGE dose higher levels of VA, SA, and 4H3MPPA were detected. The latter is possibly produced by microbial reduction of ferulic acid. Several more remarkable differences were observed between the fermentation patterns of BTE and RWGE polyphenols. After both single and continuous dosing of BTE, GA was strongly produced in the CT compartment, whereas it was markedly consumed in CD. The latter was not observed after RWGE dosing. This suggests that bacterial degradation was more effective when exposed to BTE polyphenols, possibly because of higher levels of GA and galloylated catechins in BTE. The reduction in GA in CD after BTE dosing was not accompanied by a major increase in potential GA metabolites, like 3- or 4-OMGA, pyrogallol, or hydroxylated benzoic acids. This could suggest that after BTE dosing GA is rapidly degraded down to SCFAs, for example, yielding acetate (Figure 1). Interestingly, a low production of 3- and 4-OMGA was observed, which is remarkable, since these are considered to be hepatic phase II metabolites of GA. Particularly, the increased production of 4-OMGA in CT during RWGE dosing indicates that also human gut microbiota may contribute to OMGA formation, either through bacterial O-methyltransferase activity on GA or, more likely, by bacterial cleavage of OMGA moieties, for example, from proanthocyanidins. The production of the ring-fission metabolite 3,4-dHPVL started somewhat earlier in time and colon location after BTE than after RWGE feeding, which probably reflects the higher fraction of free catechins present in tea.

Interestingly, continuous administration of BTE or RWGE polyphenols also showed markedly different effects on microbial production and metabolism of (hydroxylated) phenylpropionic and phenylacetic acids (Figure 6). Continuous BTE feeding caused elevated production of 3,4-dHPPA, 4-HPPA, and 3,4-dHPAA in CT, followed by a stronger consumption of these compounds in CD and a particularly strong production of PPA. By contrast, after RWGE feeding, 3,4-dHPPA and 3,4-dHPAA were consumed in CT, while there was a significant production of in CT and CD of monohydroxylated species 4-HPPA, 3-HPAA, and 4-HPAA but no major production of PPA. Thus the most striking difference was that, during RWGE feeding, elevated levels of particularly gallic acid and 4-HPPA, and to a lesser extent the monohydroxylated phenylacetic acids, were maintained throughout the colon, while during BTE feeding these metabolites are largely consumed in the distal colon, while particularly PPA is produced as an end product. Levels of hydroxybenzoic acids in the SHIME model remained relatively low, suggesting that in the in vivo situation β-oxidation of phenylpropionic acids to benzoic acids would mostly take place in the liver after absorption from the gut.[3,34,40]

Most phenolic acids identified in this SHIME study were also found in a previous in vitro batch fermentation study,[11] as well as in human studies testing the same BTE and/or RWGE polyphenols.[9,17,44] High variability in polyphenol fermentation patterns was observed between batches inoculated with fecal samples of different individuals. Similarly, in a human intervention study, a high interindividual variation in nutrikinetics of polyphenols and phenolic acids was observed following the consumption of BTE or RWGE,[9,44] which may be linked to marked variation in gut microbial community composition. This human study did not find significant differences between BTE and RWGE intake with respect to the average plasma nutrikinetic properties of catechins and their microbial fermentation products, including 3,4-dHPVL. It should be noted that our SHIME study was based on fecal microbiota from a single healthy human volunteer and therefore does not reflect interindividual variability. Due to the complexity of the setup, it would not be practically feasible to perform many more experiments to cover interindividual variability. While previous, simpler batch fermentation models have already indicated that interindividual variability in microbial metabolism is considerable,[11,45] the main strength of the SHIME study is its ability to study the metabolic pathways in the different colonic regions and to study repeated ingestion of polyphenols. Thus, we were able to show that microbial metabolite production and kinetics vary for different parts of the colon and are also critically dependent on the polyphenol source.

Polyphenol Catabolism Is Different in Single- and Continuous-Dose Experiments. Different polyphenol fermentation patterns were noted between the single- and continuous-dose experiments (Table 1). After the single BTE dose, 4-HPPA was produced in the CD compartment, whereas during continuous BTE dosing 4-HPPA was degraded in CD and instead a particularly strong increase in PPA production was observed. After a single dose of RWGE, 3-OMGA was degraded in CT, whereas during continuous dosing its degradation was not observed until after 8 days. Also, after the single RWGE dose, 3,4-dHPAA was produced in CT and

CD, whereas during continuous RWGE feeding degradation was observed in all vessels, starting after 3−4 days. These altered metabolic effects observed during continuous versus single-dose feeding support the view that prolonged feeding of BTE or RWGE polyphenols can cause a (slow) shift in gut microbial population/activity. Polyphenols and their microbial catabolites have been shown to exert prebiotic or selective antimicrobial effects, for example, by inhibiting the growth of certain pathogenic colon bacteria, while not affecting the growth of other, more beneficial bacteria.[8,12−14,46−49] In line with these findings, additional microbiological analysis of samples taken from the current SHIME study show that continuous dosing of BTE or RWGE may cause antimicrobial effects and may also shift the relative abundance of several biological groups (R. Kemperman et al., manuscript in preparation).

Relevance for in Vivo Effects of BTE and RWGE. The current study demonstrates that dietary source and polyphenol composition determine not only the type and levels of phenolic acids produced by the colonic microbiota but also the dynamics of their production during transit through the colon. This may have important consequences for health benefits of dietary polyphenols in vivo, as potentially high levels of phenolic acids can be produced in close proximity to the colonic epithelium.[50] Although our understanding of polyphenol metabolism and gut microbial degradation is accumulating, knowledge of local or systemic biological activities of the microbial phenolic acids remains scattered.[33,38,47,51] The traditional antioxidant activity of polyphenols may be particularly relevant in the colon because of the high local concentrations of phenolic acids.[52] A mucus layer at the luminal surface of the gastrointestinal tract potentially modulates cytotoxic effects.[53] Gut microbial phenolic acids have anti-inflammatory activity in the colon,[54−56] for example, through modulation of lipoxygenase activity[56] or by inhibition of matrix metalloproteinases as was shown for phenylvalerolactones.[57] More in vitro studies have shown that microbial phenolic acids may have antiproliferative activity in cancer cells;[58,59] may bind to and activate nicotinic acid receptor GPR109, suppressing adipocyte lipolysis;[60] and may counteract diabetic complications like protein glycation and neurodegeneration,[61] induce expression of cardiac antioxidant enzymes,[62] or act as endothelial NADPH oxidase inhibitors, thereby improving endothelial function.[63]

More research is needed to understand local and systemic bioactive properties of phenolics and to be able to formulate optimal dietary recommendations. The combined learnings of in vitro gut models and human intervention studies and the use of novel metabolomics and microbiomics technologies will contribute significantly to defining the role of the gut microbiota in modulating health effects of dietary polyphenols.[10]

In conclusion, BTE and RWGE polyphenols were catabolized into slightly different phenolic acids in the TWINSHIME gastrointestinal model. Importantly, the microbial production and degradation of metabolites was both temporally and spatially different for BTE and RWGE feeding. Moreover, continuous dosing of BTE polyphenols induced stronger changes in SCFA production, mainly acetate, than RWGE. Thus, the interaction between the food source of polyphenols and the gut microbiota is a critical determinant of polyphenol bioavailability and related potential health benefits.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Two tables, listing phenolic acid standards and phenolic composition of BTE and RWGE, and seven figures, showing temporal changes in levels of acetate, propionate, and butyrate after a single dose of BTE or RWGE; LC-MS measured kinetics of quercetin, 3,4-dHPVL, 3-OMGA, and 4-OMGA produced in the different SHIME colon compartments following single- and continuous-dose feeding of BTE or RWGE polyphenols; GC-MS-measured kinetics of various hydroxybenzoic acids and other selected phenolic acids produced in the different SHIME colon compartments following a single dose of BTE or RWGE polyphenols and of various benzoic acids during 2-week continuous dose feeding; and molecular structures of selected polyphenol precursors and microbial phenolic acids.This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

BTE, black tea extract; CA, colon ascendens; CT, colon transversum; CD, colon descendens; EGCG, (−)-epigallocatechingallate; 3,4-dHPPA, 3-(3,4-dihydroxyphenyl)propionic acid; 3M4HPPA, 3-(3-methoxy-4-hydroxyphenyl)propionic acid; 3-HPPA, 3-(3-hydroxyphenyl)propionic acid; 4-HPPA, 3(4-hydroxyphenyl)propionic acid; PPA, 3-phenylpropionic acid; 3,4-dHPAA, 2-(3,4-dihydroxyphenyl)acetic acid; 3-HPAA, 2-(3-hydroxyphenyl)acetic acid; 4-HPAA, 2-(4-hydroxyphenyl)acetic acid; 3,4-dHBA, 3,4-dihydroxybenzoic acid; 3-HBA, 3-hydroxybenzoic acid; 4-HBA, 4-hydroxybenzoic acid; VA, vanillic acid; HVA, homovanillic acid; SA, syringic acid; 3,4-dHPVL, (−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; 3M4HPVL, (−)-5-(3′-methoxy-4′-hydroxyphenyl)-γ-valerolactone; 3-OMGA, 3-*O*-methylgallic acid; 4-OMGA, 4-*O*-methylgallic acid; RWGE, red wine−grape juice extract; SCFA, short-chain fatty acid; SHIME, simulator of the human intestinal ecosystem

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