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Profiling of Phenols in Human Fecal Water after Raspberry Supplementation

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The phenolic compositions of fecal water samples from ten free-living human subjects without marked dietary restrictions were monitored before and after intake of raspberry puree (200 g/day, 4 days) using gas chromatography−mass spectrometry. No single phenolic component was increased in all subjects after intake, but a majority of subjects had significant elevations in phenylacetic acid (7/10), 4-hydroxyphenylacetic acid (6/10), 3-hydroxyphenylacetic acid (5/10), 3-phenylpropionic acid and 3-(4-hydroxyphenyl)propionic acid. The levels of 3,4-dihydroxbenzoic acid were elevated in 8/10 subjects, significantly for 6 subjects ($p < 0.05$), and not significantly reduced in the other 2 subjects. In addition, unlike most other fecal metabolites, the increase was always >2-fold. This metabolite may be representative of the increased colonic dose of cyanidin anthocyanins. The colonic microbiota varied greatly between individuals, and supplementation with raspberries did not produce any statistically significant alterations in the profile of colonic bacteria, nor was a common pattern revealed to account for the interindividual variations observed in the fecal water phenolic profiles.

KEYWORDS: Raspberries; fecal water; microbiota; phenolics; anthocyanins

INTRODUCTION

The ingestion of fruit and vegetables (FAV) has been positively correlated with reduced incidence of cardiovascular disease, cancers and other chronic health disorders (*1*). Many hypotheses have been mooted for the putative protective effect, and one of the most popular and persistent is that phytochemicals from FAV prevent damage caused by free radicals which are implicated in disease states (*2*)

Berries specifically are particularly rich in polyphenol antioxidants, for example raspberries can consistently provide ∼200 mg of polyphenols/100 g of fruit (*3*), but the hypothesis that systemic polyphenols can influence cellular events is contradicted by the low serum bioavailability of polyphenols and the variation of *in vivo* stability and metabolism for different polyphenol classes, which influences circulating serum levels (*4*). Similarly, studies on the levels of polyphenols, or their metabolites, in urine also show a mixed response suggesting that differences in the stability and metabolism of polyphenol classes influence their clearance (*5*, *6*). However, certain metabolites (such as the urolithins) have been identified that seem to be markers for ingestion and clearance of dietary ellagitannins (*7*). These compounds are formed through colonic fermentation of ellagic acid obtained from breakdown of dietary ellagitannins (*8*).

Indeed, it is now recognized that a large portion of dietary polyphenols are retained within the gastrointestinal tract [e.g., see studies on ileostomy patients (*9*)] and reach the colon. Once in the colon, berry polyphenols are subject to the action of fermentative microbiota and subsequent degradation (*10*, *11*). These metabolites and/or undegraded and unabsorbed polyphenols may play a role in the defense of the colon through direct interaction with the colonic epithelium. Indeed, *in vitro* studies on colon cancer cell lines have reported putative anticancer activities using berry extracts (*12*, *13*).

The aqueous phase of human feces (namely, fecal water) will more readily interact with the colonic epithelium than the solid phase and therefore may have the capacity to directly affect pathogenesis in the colon. Indeed, fecal water has been reported to be a highly variable bioactive material, able to elicit a range of cellular effects including DNA damage and cytotoxicity (*14*−*16*), and this bioactivity is subject to alteration by dietary means [as reviewed recently (*17*)]. A previous study also reported a positive correlation between dietary consumption of phenolics and an increased antioxidant capacity of fecal water in healthy subjects (*18*).

Considering the retention of polyphenols in the gastrointestinal tract, it is also possible that fecal water could contain

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biomarkers for berry consumption. To date, only a few papers have reported on the phenolic profiles of fecal water (*19*, *20*), and there is a paucity of data in relation to dietary polyphenol consumption on the phenolic constituents of the fecal water. This paper reports on the effect of raspberry supplementation on fecal phenolic profile in free-living human volunteers with few dietary restrictions.

METHODS

Materials. Raspberries (*Rubus ideaus* variety Glen Ample) were purchased from farmers in Angus, Scotland. To ensure homogeneity and consistency, fresh raspberry puree was produced in a single batch using appropriate food grade facilities at SCRI, individually portioned (200 g) and frozen before transport to the University of Ulster (Northern Ireland).

Subjects and Study Design. The study was carried out with 10 male volunteers (mean age 38 ± 9.8 years, range 27−60 years, mean BMI 27 ± 3.05). All subjects were apparently healthy omnivores, nonsmokers and nonusers of dietary supplements or medications. The study was conducted with the prior approval of the ethics committee of the University of Ulster and with the informed consent of participants.

The study was designed to determine whether consistent changes in fecal water phenolic profiles could be observed from subjects consuming raspberries under normal free living conditions. Restrictions in diet may standardize the detection of metabolites by reducing intersubject variation. However, this study was focused on assessing changes in a free living population with the fewest restrictions on their normal diet or lifestyle. The pilot study required 10 male subjects to consume raspberry puree daily for 4 days in addition to their habitual diet. Fasting subjects consumed a 200 g raspberry puree (single dose, sealed sachet) for 4 days consecutively without accompaniment (except water ad lib), in the presence of an investigator to ensure compliance. Subjects were instructed to maintain their habitual diet for the duration of the supplementation period. A 4-day food diary (day T0−T3) was completed to assess dietary intake during supplementation. Single stool samples were collected from each subject, just prior to (day T1) and after the 4-day supplementation period (day T4) and were stored at 4 °C for up to 2 h before processing. The fecal sample was weighed mixed 1:1 w/v with ice cold PBS then homogenized in a Seward 600 stomacher for 2 × 2 min cycle. The fecal slurry was then ultracentrifuged at 50000*g* for 2 h at 4 °C. The supernatant was removed and filter sterilized (0.22 μm) on ice before the fecal water was aliquoted for storage at −80 °C. Additionally, fresh stool samples (5 g per tube) were stored at −80 °C for subsequent microbiota composition analysis. All analyses were carried out at the end of the intervention period, and the researchers were blinded to these samples during analyses.

Analysis of Raspberry Polyphenol Content by Liquid Chromatography−Mass Spectroscopy (LC−MS^n). Raspberry puree was extracted with an equal volume of acetonitrile containing 0.2% (v/v) formic acid for 30 min in the dark with rotary shaking (200 rpm). After centrifugation (10000*g*, 10 min, 4 °C), the extract was removed and stored at −20 °C. Phenol and anthocyanin contents were measured as before (*3*). The polyphenol composition was assessed using liquid chromatography−mass spectroscopy (LC−MS^n) and was essentially identical to previous reports (e.g. ref *21* and references therein).

Analysis of Phenolic Compounds in Fecal Water. Fecal water samples were thawed, centrifuged (15000*g*, 5 min, 4 °C) and samples removed for analysis of phenol content by the Folin method as described previously (*3*). For GC−MS analysis, triplicate fecal water samples (1 mL) were acidified with 20 μL 10% (v/v) formic acid in ultra pure water then extracted with 2 × 1 mL of ethyl acetate. In brief, the ethyl acetate−fecal water samples were vortex mixed for 3 × 30 s and centrifuged in a microfuge for 4 min at 4 °C, and the ethyl acetate fraction was removed to a new tube. The extraction procedure was repeated and the combined ethyl acetate fraction dried in reaction vials preloaded with 5 nmol of the internal standards (6-methylflavone, 2,4,5-trimethoxycinnamic acid and 2-hydroxy-5-methoxybenzoic acid). Previous studies (*19*) used ethyl acetate extraction and then solid phase extraction on diatomaceous earth, but we found that this procedure did not improve sample quality or recovery. Similarly, treatment with *Helix pomatia* extracts to hydrolyze sulfated or glucuronidated derivatives did not increase the yield or alter the composition of phenolic compounds in agreement with the findings of Grun et al. (*22*).

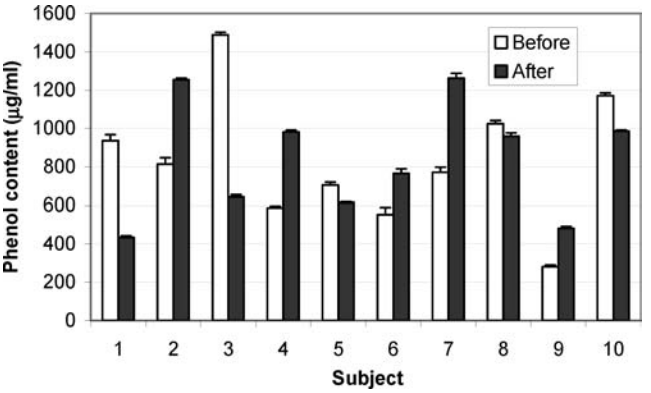

Figure 1. Phenol content of fecal water samples before and after raspberry supplementation. All values are averages of triplicate assays ± standard errors and are expressed as gallic acid equivalents.

The derivatization and GC−MS procedure essentially replicated a previous method (*19*) with minor changes due to hardware differences. The dried fecal water ethyl acetate fraction contained internal standards of 6-methylflavone, 2,4,5-trimethoxycinnamic acid and 2-hydroxy-5-methoxybenzoic acid. The dry sample was derivatized with 10 μL of acetonitrile and 50 μL of BSTFA and 1% TMCS for 4 h at 50 °C. These conditions offered effective derivatization of the standard phenolics without reducing the 6-methylflavone signal, which is not derivatized and can be used to judge recovery. 75 standard phenolic compounds were run as standards (as ref *19*) to aid identification of fecal phenolics by comparison of retention time, m/z and MS^2 properties.

Derivatized samples were analyzed using a Thermo Finnigan Trace DSQ quadropole GC−MS system equipped with an autosampler. Data were acquired and analyzed using the XcaliburTM software package V. 1.4. The MS source and GC−MS interface were kept at 230 and 290 °C, respectively. Separations were carried out on a fused silica capillary column (Agilent J&W DB-5MS, 15 m × 0.25 mm × 0.25 μm). Helium carrier gas was applied at 1.5 mL/min. Derivatized samples (1 μL) were injected into the GC injection port using a split ratio of 16:1. The column temperature gradient was t = 0, 100 °C; t = 1.5 min, 100 °C, t = 5.5 min, 140 °C; t = 7.5 min, 140 °C; t = 8.2 min, 150 °C; t = 13.2 min, 160 °C; t = 17 min, 215 °C; t = 19.5 min, 262 °C; t = 23.5 min, 262 °C; t = 26.5 min, 290 °C and t = 29.5 min, 290 °C.

Mass spectra were acquired over the mass range 35−900 amu at 6 scans s^{-1} under EI positive ionization conditions at 70 eV, after a solvent delay of 1.5 min with the ion source maintained at 230 °C. Full scanning MS was initially performed for each standard to compile a database of EI spectra.

Positive identification of compounds in fecal water was made after comparison with retention time and mass ion ratios of standards. Quantification was achieved by relating the peak area of the compound with the internal standard peak area, and data were expressed as mean ± standard deviation. Calibration curves for the standard phenolics were constructed using 5 concentrations (0.25−100 nmol) in triplicate and showed linearity above r^2 = 0.95. The method used gave essentially similar results to the methodology reported previously (*19*, *22*). Variations in specific phenolic concentrations in fecal water samples before and after supplementation were assessed for statistical significance using a two tailed paired t test with $P > 0.05$.

Microbiota Composition. *DNA Extraction and PCR Amplification of the 16S rRNA Gene*. Frozen fecal samples were thawed on ice before DNA extraction. DNA was isolated using FastDNA Spin Kit for Soil (Qbiogene, Cambridge, U.K.) according to the manufacturer's instruction. DNA was used as a target for amplification of approximately 200 bp of the variable V3 region of the 16S rRNA (rRNA) gene corresponding to positions 341−534 using the primers P2 (5′-ATTACCGCG GCTGCTGGCTGG-3′) and P3 (5′CGCCCGCCGCGCGCGGCGGGC-GGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′) (*23*). The program used for the amplification was a touch down PCR: an initial denaturation at 95 °C for 5 min, followed by 20 cycles at 95 °C for 1 min, 65 °C for 1 min and 72 °C for 1 min, going down after every two cycles 1 degree in the annealing temperature (from 65 to 56 °C) and 8 cycles at 95 °C

Table 1. Changes in Fecal Phenolic Metabolites before and after Supplementation[a]

compound		subject 1	S2	S3	S4	S5	S6	S7	S8	S9	S10
phenylacetic acid	before	268.89	55.21	348.63	166.390	55.96	30.47	646.65	231.61	80.33	95.13
	SD	40.97	10.45	13.65	14.675	3.94	2.67	24.88	22.55	3.51	0.21
	After	**523.024**** [b]	**175.68****	**229.69**	**223.997****	**194.67****	**161.92****	372.10	86.57	**104.79****	**151.63****
	SD	10.13	15.59	21.83	10.83	21.145	38.97	30.47	7.18	1.67	5.71
4-hydroxyphenylacetic acid	before	4.60	2.06	7.21	26.07	7.64	51.27	3.43	6.34	8.23	11.15
	SD	0.18	0.21	0.28	1.46	0.18	1.26	0.12	0.44	0.11	0.028
	after	**7.27****	**3.46****	**6.59**	**30.39***	**9.13****	14.58	**4.25***	**3.39**	**49.69****	7.90
	SD	0.48	0.23	0.20	1.42	0.02	0.41	0.17	0.42	0.61	0.02
3-hydroxyphenylacetic acid	before	10.82	1.71	85.92	5.03	51.62	7.93	6.53	58.54	27.84	3.06
	SD	0.96	0.08	2.97	0.35	2.51	0.10	0.29	5.26	0.82	0.14
	after	11.24	**3.60****	41.34	**7.29****	**103.92****	**18.85****	**15.84****	3.42	13.53	2.04
	SD	0.36	0.15	0.46	0.18	6.19	0.54	0.27	0.07	0.07	0.14
3,4-dihydroxyphenylacetic acid	before	2.01	0.21	0.25	27.70	0.50	2.10	1.88	0.11	2.59	74.33
	SD	0.07	0.07	0.08	1.37	0.01	0.10	0.01	0.11	0.09	3.85
	after	1.91	1.87	0.07	3.55	0.08	2.02	1.95	**3.19***	**3.42***	7.04
	SD	0.025	0.006	0.071	0.390	0.025	0.072	0.036	1.509	0.165	0.559
3-phenylpropionic acid	before	148.81	9.77	167.551	41.78	105.86	70.01	208.44	201.30	0.16	3.23
	SD	21.13	1.24	14.56	1.72	8.63	0.16	7.68	12.36	0.02	0.01
	after	**216.80****	**15.58****	**124.63**	**45.34***	**271.57****	**162.53****	170.39	30.77	**0.22***	2.03
	SD	6.81	0.60	9.56	0.14	13.63	17.70	6.38	2.11	0.021	0.01
3-(4-hydroxyphenyl)propionic acid	before	1.84	0.07	3.54	58.14	1.12	0.58	1.26	0.84	16.08	69.64
	SD	0.28	0.04	0.03	0.42	0.04	0.07	0.11	0.03	0.64	0.87
	after	1.14	**0.21****	**1.41**	**172.46****	**4.16****	**2.50****	0.55	0.16	**90.46****	46.34
	SD	0.09	0.04	0.02	10.15	0.28	0.01	0.03	0.04	2.07	0.02
benzoic acid	before	4.71	3.42	5.24	3.01	5.60	5.89	2.62	4.34	1.60	8.64
	SD	1.03	1.27	0.47	0.28	0.14	1.68	0.33	0.92	0.20	0.50
	after	2.96	4.82	5.93	3.27	**8.97***	7.90	2.39	2.78	1.90	2.67
	SD	0.60	0.18	0.57	0.41	1.81	0.62	0.46	0.69	0.44	0.73
salicylic acid	before	0.10	0.20	0.21	0.19	0.13	0.75	0.16	0.14	0.17	0.20
	SD	0.07	0.01	0.03	0.11	0.01	0.08	0.04	0.02	0.01	0.04
	after	0.09	0.15	0.25	0.11	**0.27****	**8.42****	0.04	0.10	0.20	0.13
	SD	0.02	0.01	0.01	0.01	0.01	0.38	0.01	0.02	0.01	0.04
4-hydroxybenzoic acid	before	0.84	0.12	4.63	1.70	4.47	4.85	1.31	4.87	1.98	10.63
	SD	0.13	0.063	0.021	0.34	0.03	0.31	0.02	0.03	0.12	0.30
	after	**1.13***	1.39	4.52	0.80	4.52	2.16	1.29	4.57	**12.03****	6.04
	SD	0.02	0.04	0.04	0.04	0.03	0.25	0.01	0.05	0.25	0.09
3,4-dihydroxybenzoic acid	before	0.17	0.22	0.18	0.24	0.44	0.86	0.10	0.16	0.31	1.11
	SD	0.02	0.02	0.01	0.08	0.23	0.29	0.05	0.09	0.01	0.01
	after	**0.97****	**0.48***	0.29	0.50	**2.04****	**0.34**	**0.73****	**0.52****	**1.78****	0.81
	SD	0.03	0.14	0.08	0.17	0.51	0.26	0.05	0.02	0.37	0.09
2,5-dihydroxybenzoic acid	before	2.29	1.18	1.12	4.44	4.93	4.51	1.29	4.09	1.46	2.15
	SD	0.28	0.01	0.09	1.85	0.03	1.37	0.10	0.07	0.24	0.10
	after	2.31	1.24	**2.20****	3.46	3.14	3.21	**1.76***	0.15	3.41	1.31
	SD	0.27	0.08	0.12	1.13	0.14	0.10	0.19	0.02	1.40	0.01
4-methoxybenzoic acid	before	0.011	0.014	0.050	0.015	0.010	0.028	0.011	0.014	0.025	11.470
	SD	0.020	0.002	0.023	0.012	0.006	0.014	0.009	0.009	0.002	0.576
	after	0.008	0.021	0.002	0.017	0.019	0.005	0.015	0.009	0.015	2.644
	SD	0.012	0.006	0.008	0.007	0.002	0.008	0.010	0.014	0.002	0.731
isoferulic acid	before	0.54	0.24	0.87	0.46	0.41	0.25	0.43	0.67	0.16	1.11
	SD	0.17	0.18	0.01	0.07	0.09	0.05	0.20	0.33	0.04	0.26
	after	0.07	0.96	0.98	0.26	**1.04****	0.19	0.63	0.98	0.60	0.62
	SD	0.09	0.16	0.24	0.10	0.10	0.22	0.14	0.10	0.30	0.10
caffeic acid	before	3.65	3.71	8.99	3.43	7.78	3.38	3.61	8.09	3.00	10.78
	SD	0.08	0.28	0.29	0.30	0.43	0.13	0.25	0.24	0.11	0.90
	after	3.64	**5.07****	7.68	3.37	8.12	3.79	4.00	9.04	2.75	7.89
	SD	0.20	0.29	0.06	0.05	0.07	0.18	0.21	0.04	0.06	0.07
sinapic acid	before	0.014	0.067	0.143	0.052	0.012	0.038	0.058	0.114	0.006	0.465
	SD	0.002	0.023	0.043	0.036	0.003	0.039	0.032	0.039	0.038	0.028
	after	**0.041****	0.151	0.083	0.064	0.095	0.021	0.034	0.060	**0.175***	0.079
	SD	0.002	0.049	0.024	0.037	0.059	0.024	0.027	0.061	0.067	0.025
naringenin	before	6.00	1.20	7.24	4.17	2.72	2.00	1.36	0.42	0.07	0.90
	SD	0.97	0.77	2.44	1.02	2.54	1.33	1.68	0.05	0.06	0.01
	after	1.79	3.27	10.35	1.01	1.42	1.21	2.27	0.64	**1.11***	0.43
	SD	1.95	1.06	3.06	0.59	0.40	0.30	0.57	0.30	0.22	0.04
hesperetin	before	0.71	0.65	0.18	0.29	0.01	0.05	0.35	0.06	0.06	7.93
	SD	0.60	0.57	0.06	0.15	0.03	0.08	0.44	0.04	0.01	0.96
	after	0.32	0.69	0.17	0.52	0.03	0.42	0.62	0.17	0.07	0.14
	SD	0.18	0.35	0.10	0.72	0.04	0.06	0.60	0.24	0.20	0.08

Table 1. Continued

compound		subject 1	S2	S3	S4	S5	S6	S7	S8	S9	S10
epicatechin	before	6.08	2.87	21.11	2.50	0.88	3.02	3.22	0.23	2.46	5.19
	SD	0.79	0.13	1.96	0.10	0.12	0.16	0.19	0.01	0.16	0.05
	after	4.40	**3.54****	3.58	2.45	**2.45****	2.94	3.33	**1.09***	2.53	2.77
	SD	0.35	0.18	1.93	0.06	0.65	0.19	0.31	0.44	0.10	0.38
catechol	before	0.16	0.34	0.87	1.59	1.48	0.20	0.06	0.73	0.12	3.42
	SD	0.04	0.01	0.02	0.10	0.08	0.12	0.05	0.14	0.03	0.29
	after	0.13	0.18	0.54	0.98	0.65	**1.36****	0.08	0.46	**0.97****	0.75
	SD	0.07	0.03	0.07	0.09	0.01	0.20	0.06	0.12	0.07	0.07
trihydroxybenzene	before	0.002	0.002	0.026	0.116	0.027	0.004	0.003	0.027	0.073	0.033
	SD	0.003	0.001	0.002	0.006	0.001	0.002	0.002	0.000	0.002	0.002
	after	0.001	0.001	**6.520****	**0.250****	0.024	0.002	0.006	0.027	**0.463****	0.027
	SD	0.001	0.001	0.126	0.041	0.001	0.001	0.004	0.004	0.057	0.001

[a] All figures are expressed as μg/mL concentrations in the fecal water with standard deviations from three measurements. [b] Values that increase significantly after supplementation are highlighted in bold. (Two-tailed paired *T* test, $^{*}P = 0.05$ to 0.001, $^{**}P < 0.001$.

for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final extension period at 72 °C for 3 min. The resulting amplicons were visualized on a 1.5% (w/v) TAE buffer (National Diagnostics, U.K.) agarose gel to check for PCR products within the predicted size range (200−250 bp).

DGGE. PCR fragments were separated by denaturing gradient gel electrophoresis (DGGE) consisting of 8% polyacrylamide, performed using the BDH system from VWR International Ltd. (UK), following the manufacturer's guidelines. PCR products (6 μL) were loaded onto 8% (w/v) TAE polyacrylamide gels (40 mmol/L of Tris base, 20 mmol/L of acetic acid and 1 mmol/L of EDTA, pH 8.3), which contained a 40−60% denaturant gradient (100% denaturant, 7 mol/L of urea and 40% (v/v) deionized formamide). Electrophoresis was performed at a constant voltage of 100 V and at a constant temperature of 60 °C for 16 h. Gels were then stained with $AgNO_3$ as described previously (*24*), and the gel image was scanned using Canon Scanning software (Canon Scanner Lide 50, Surrey, U.K.). Scanned DGGE images were analyzed by Quantity One software (Version 4.5.2, Bio-Rad Laboratories, Herts, U.K.) to generate a dendogram of similarity between DGGE profiles.

RESULTS

Each 200 g portion of raspberry puree contained 296 ± 14 mg gallic acid equivalents of total phenols and 40.3 ± 2.3 mg cyanidin glucoside equivalents of total anthocyanins. The raspberry sample gave a polyphenol composition by LC−MS that was essentially similar to our previous extracts (*21*) but with higher ellagitannin content and smaller amounts of flavonols and hydroxycinnamic acid derivatives.

All ten subjects completed the raspberry supplementation study; three subjects however did not return a completed food diary. The subjects did not alter their habitual diet over the 4-day period in response to raspberry supplementation and, in particular, they did not alter their intake of polyphenol-rich beverages or fruit and vegetables. They consumed approximately the recommend dietary allowance (RDA) for protein and fiber [88 g/day and 18 g/day respectively] but were below the RDA for fat and carbohydrate [95 g/day and 275 g/day respectively]. The average group presupplementation stool weight was 108.6 ± 72.9 g and stool pH was 7.3 ± 0.3; postsupplementation average group stool weight was 92.5 ± 40.2 g and stool pH was 7.4 ± 0.3.

The phenol content of the fecal water samples, as determined by the Folin method, increased significantly in 5 out of 10 subjects over the sampling period (**Figure 1**), decreased significantly in 3 subjects and was unchanged in 2 subjects. However, as the Folin method detects redox-active compounds (*25*), this does not necessarily correlate with phenolic content.

One hundred sixteen peaks were consistently found in the fecal water samples from all ten subjects. Specific components could also be identified in the before or after samples from individual subjects but were not identified in other subjects. Of the 116 peaks, 50 compounds could be unambiguously identified by comparison with standards or by matching MS data to spectral databases. A further 25 were putatively identified, and 41 could not be matched to any component. Of the identified compounds, the major components were lipid or bile acid derivatives (*24*), phenolic derivatives (*20*) and nitrogen-containing heterocyclic compounds (*6*). It is possible that other GC−MS methods could have identified more components (*26*).

The major phenolic components were phenylacetic acid and phenylacetic acid derivatives, followed by phenylpropionic acid derivatives with smaller amounts of cinnamic acid and benzoic acid derivatives (**Table 1**). This agrees with previous work (*19*, *22*, *26*). Some phenolic components were significantly increased in the majority of subjects after raspberry intake, i.e. phenylacetic acid (7/10), 3,4-dihydroxyphenylacetic acid (6/10), 3-hydroxyphenylacetic acid (5/10) but others, such as 4-hydroxyphenylacetic acid, were only increased in one of the subjects. Increases were not consistently conserved within a class of products. For example, increased levels of other phenylacetic derivatives were not consistently seen across the subjects with increased levels of phenylacetic acid.

The levels of phenylpropionic acid derivatives were also influenced by raspberry intake with 6/10 subjects showing significant increases in 3-phenylpropionic acid and 5/10 in 3-(4-hydroxyphenyl)propionic acid. Once again, the subjects who showed increased levels of one phenylpropionic derivative did not show increased levels of the other phenylacetic derivatives or, indeed, of phenylacetic acid derivatives.

Benzoic acid derivatives were generally present at 5−10-fold lower levels (**Table 2**) than the phenylacetic and phenylpropionic acids. Benzoic acid was significantly increased in 1 subject; 4-hydroxybenzoic acid was also significantly increased in 1 subject but down or unchanged in 5 other subjects. 2,5-Dihydroxybenzoic acid was increased in 3 subjects and reduced or unchanged in 7 subjects. Salicylic acid (2-hydroxybenzoic acid) was identified in all subjects and was significantly increased in two subjects. Most relevantly, 3,4-dihydroxybenzoic acid was increased in 8 subjects (6 significantly) and reduced in 2 subjects. However, these reductions were not significant.

The cinnamic acid derivatives, isoferulic and caffeic acid, were increased in 1 subject out of 10 subjects, but sinapic acid was only present in small amounts (< 0.5 ng/mL) and was increased in 2 subjects following supplementation. Naringenin and hesperetin could be detected in all subjects, which confirms the ubiquity of citrus products in the diet.

Those phenolic compounds which rose after raspberry supplementation in some subjects (**Table 1**) did not often markedly

Table 2. Summary Table of Changes in Fecal Phenolic Components

compound	N[a]	concn (μM) low	concn (μM) high	X fold range
phenylacetic acid	7	224.2	4754.4	21.2
4-hydroxyphenylacetic acid	6	13.8	327.0	23.7
3-hydroxyphenylacetic acid	5	11.2	683.6	61.1
3,4-dihydroxyphenylacetic acid	2	0.6	442.3	675.5
3-phenylpropionic acid	6	1.1	1810.0	1696.9
3-(4-hydroxyphenyl) propionic acid	5	0.4	1038.6	2462.9
benzoic acid	1	13.1	73.5	5.6
salicylic acid	2	0.3	60.9	210.0
4-hydroxybenzoic acid	2	0.9	87.0	100.0
3,4-dihydroxybenzoic acid	6	0.6	13.2	20.4
2,5-dihydroxybenzoic acid	2	1.0	32.0	32.9
4-methoxybenzoic acid	0	0.06	75.7	1150.0
isoferulic acid	1	0.8	5.4	6.5
caffeic acid	1	16.7	60.0	3.6
sinapic acid	2	0.04	2.1	47.0
naringenin	1	0.3	37.9	147.1
hesperetin	0	0.04	26.2	607.7
epicatechin	3	0.80	21.3	26.4
catechol	2	0.45	30.9	68.0
trihydroxybenzene	3	0.008	51.6	6500.0

[a] Number of subjects with significant increases ($P = 0.05$ to 0.001).

exceed presupplementation levels in other subjects (**Table 2**). Exceptions included 3-hydroxyphenylacetic acid, 3-(4-hydroxyphenyl)propionic acid, trihydroxybenzene and 3,4-dihydroxybenzoic acid. It was notable that the minimum increase of 3,4-dihydroxybenzoic acid following supplementation was 2-fold and the maximum was 7-fold and in no subject was any decrease statistically significant.

Previous work has proven that the DGGE profiling can discriminate interindividual variation in colonic microbiota and can identify changes in microbiota communities due to perturbation of diet and/or probiotic treatments (*27*, *28*). Indeed, DGGE profiling before supplementation with raspberry puree revealed considerable interindividual variation in the composition of colonic microbiota between subjects (see Supporting Information). However, although some small changes to the colonic microbiota profile after consumption of the raspberry puree were observed, principal component analyses confirmed that the 4-day raspberry supplementation did not significantly and consistently alter the microbiota composition (results not shown). Analysis of the microbiota profiles and the metabolite profiles observed in the fecal water did not reveal any common patterns which could be used to correlate specific microbiota alterations to the presence and absence of certain metabolites.

DISCUSSION

Studies have highlighted the putative chemoprotective effect of berry phytochemicals (e.g. ref *29*,) but the fate of these components in the colon is less understood. This study revealed large interindividual variability in the phenolic profiles of fecal water samples after supplementation with raspberries (**Table 2**). Of the twenty phenolic components identified, no single phenolic component was significantly increased in all subjects after raspberry intake, but the majority of subjects had significant elevations in phenylacetic acid (7/10), 4-hydroxyphenylacetic acid (6/10), 3-hydroxyphenylacetic acid (6/10), 3-(4-hydroxyphenyl)propionic acid (5/10) and 3,4-dihydroxybenzoic acid (6/10). The concentrations of all 20 phenolic components detected varied noticeably between subjects. This study was supplementary in nature and did not entail a controlled dietary regime; however data from food diaries indicated no alteration in diet over the 4-day supplementation period, in particular, no intake of berries or increases in other polyphenol-rich foods or beverages. Therefore, it seems probable that the changes in fecal phenolics were a response to berry supplementation, but that individuals responded differently to supplementation.

Interindividual variation in phenolic profiles has been noted previously. For example, *in vitro* incubation of blueberry samples with human fecal slurries produced phenolic-rich fermentation products that differentially altered prostanoid production in CCD-Co18 cells (*30*). The fermentation products from one individual reduced while another increased prostanoid production. These samples were significantly different in their content of 8 of the 26 measured phenolic compounds. Most notably, those fermentation products that increased prostanoid levels had substantially higher levels of 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, 3-methoxy-4-hydroxyphenylacetic acid and 4-(hydroxyphenyl)-propionic acid.

The majority of fecal water samples from vegetarians (16 out of 19) significantly reduced prostaglandin production (PGE_2) in TNFα stimulated HT 29 cells (*31*). The metabolic profiles obtained by NMR analysis differed in their abundance of amino acids, short chain fatty acids and phenolic components, such as 3-phenylpropionic acid which varied 5-fold in relative NMR intensity. In addition, 3-hydroxyphenylacetic acid and 4-hydroxybenzoic acid were only detected in some fecal water samples and varied 2−3-fold between individuals. Indeed, a range of similar colonic phenolic metabolites have been shown to have anti-inflammatory activity in different model systems (*32*). Large interindividual variation in fecal phenolic content was also noted in a recent NMR study examining changes in the metabolite profile in response to grape and/or wine extract supplementation, where no change was observed when grape extract was used (*20*). The authors concluded that different colonic microbiota have common biochemical characteristics which results in different substrates being metabolized to a specific metabolic pattern. Similarly high interindividual variation in fecal water composition was also reported in another NMR-based study which focused on discrimination of fecal water samples from normal and colon cancer patients (*33*).

There could be many reasons for the large interindividual variation in fecal response to this polyphenol supplementation. The subjects did not follow a restricted dietary regime, so the effects of raspberry supplementation were overlaid on differences in dietary habits. Humans also have different gastrointestinal transit times, and the length of residence in the colon could influence the effectiveness of colon microbiota-catalyzed transformation [which has been shown to influence enterolactone production from lignans (*34*)]. In addition, the extent of interindividual variation in enterohepatic circulation is not well documented and could influence fecal phenolic composition (*35*). However, the main consideration is likely to be the well-known interindividual variation in colonic microbiota composition (see ref *36*) which may be influenced by diet, lifestyle and genetics (*37*).

A range of dietary phenolics appear to have the ability to alter microbiota composition *in vitro* as demonstrated by either prebiotic (*38*) or antipathogenic activity (*39*). However, 4-day raspberry consumption (and the associated colonic fermentation of phenolics) did not alter the microbiota composition in a characteristic or statistically significant manner. Principal component analysis revealed no correlation between the microbiota compositions and the presence or absence of metabolites (results not shown). This may reflect differences in complexity between *in vitro* and *in vivo* modeling and/or the short duration of the supplementation period used in the study. Prebiotic supplementation commonly requires a 2−4 week period before consistent

observable changes are reported in the subject microbiota composition (*40*), but, on the other hand, long-term supplementation with polyphenol-rich black tea did not markedly alter fecal microbiota profiles even in subjects with otherwise tightly controlled diets (*41*).

Studies using incubation with fecal inocula under simulated colonic conditions have provided valuable information about the time scale and mechanisms of microbial tranformation on pure polyphenol compounds (*11*,*35*) or anthocyanin-rich extracts (*42*). The main polyphenol components of raspberry are anthocyanins and ellagitannins, with smaller amounts of flavonols and hydroxycinnamic acid derivatives (e.g. ref *21*). It has been established that ellagitannins are broken down by colonic microbiota via ellagic acid to urolithin derivatives, which are excreted via the urine after reabsorption through the colonic epithelia into the bloodstream (*8*). Urolithin derivatives are probably present in the feces in this study but could not be detected by GC−MS, as they are unstable to derivatization.

Under controlled fermentation with fecal inocula, cyanidin anthocyanins, which are the major anthocyanin constituents in raspberry (*43*), may be expected to be deglycoslyated to cyanidin and then metabolized into 3,4-dihydroxybenzoic acid (*11*,*35*) and phenylacetic acid derivatives by C ring fission. A consensus has developed that hydroxybenzoic acids derived from their B-ring would be major intestinal metabolites of dietary anthocyanins (*44*), and recent work has confirmed urinary excretion of benzoic acids following berry intake (*45*, *46*). Hydroxybenzoic metabolites are candidate biomarkers for anthocyanins whereas (say) phenylacetic derivatives may be produced by the degradation of other common polyphenols (*47*). However, it should be noted that 3,4-dihydroxybenzoic acid was increased in rat feces after supplementation with the xanthone, norathyriol (*48*). Nevertheless, 3,4-dihydroxybenzoic acid was identified as a major metabolite of cyanidin glucoside in human feces after a single supplement of 70 mg cyanidin glucoside in six fasting subjects (*46*). Although the fecal increases in 3,4-dihydroxybenzoic acid noted in this study were smaller than reported by Vitaglione et al. (*46*), these changes were identified in essentially free-living subjects with no dietary restrictions from a considerably smaller daily dose over a 4-day supplementation period.

The great interindividual variation in colonic microbiota makes identification of candidate biomarkers difficult, but it is intriguing that Puuponen-Pimiä et al. (*49*) have also reported elevated levels of 3,4-dihydroxybenzoic acid in human feces following long-term supplementation with cyanidin anthocyanin-rich bilberries.

To conclude, raspberry supplementation at 200 g/day (the equivalent of 2.5 UK portions) for 4 days did not cause consistent changes in the fecal phenolic composition in 10 free living subjects. However, the supplementation produced a notable increase in 3,4-dihydroxybenzoic acid, which may be representative of the increased colonic dose of cyanidin anthocyanins. At the same time, the supplementation did not produce any significant changes in the profile of colonic bacteria.

Supporting Information Available: LC−MS traces of raspberry samples, DGGE profile, and tables of average daily nutrient intake and putative identification of major raspberry components. This material is available free of charge via the Internet at http://pubs.acs.org.

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