

Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study^{1–3}

Xenofon Tzounis, Ana Rodriguez-Mateos, Jelena Vulevic, Glenn R Gibson, Catherine Kwik-Urbe, and Jeremy PE Spencer

ABSTRACT

Background: The absorption of cocoa flavanols in the small intestine is limited, and the majority of the flavanols reach the large intestine where they may be metabolized by resident microbiota.

Objective: We assessed the prebiotic potential of cocoa flavanols in a randomized, double-blind, crossover, controlled intervention study.

Design: Twenty-two healthy human volunteers were randomly assigned to either a high-cocoa flavanol (HCF) group (494 mg cocoa flavanols/d) or a low-cocoa flavanol (LCF) group (23 mg cocoa flavanols/d) for 4 wk. This was followed by a 4-wk washout period before volunteers crossed to the alternant arm. Fecal samples were recovered before and after each intervention, and bacterial numbers were measured by fluorescence in situ hybridization. A number of other biochemical and physiologic markers were measured.

Results: Compared with the consumption of the LCF drink, the daily consumption of the HCF drink for 4 wk significantly increased the bifidobacterial ($P < 0.01$) and lactobacilli ($P < 0.001$) populations but significantly decreased clostridia counts ($P < 0.001$). These microbial changes were paralleled by significant reductions in plasma triacylglycerol ($P < 0.05$) and C-reactive protein ($P < 0.05$) concentrations. Furthermore, changes in C-reactive protein concentrations were linked to changes in lactobacilli counts ($P < 0.05$, $R^2 = -0.33$ for the model). These in vivo changes were closely paralleled by cocoa flavanol-induced bacterial changes in mixed-batch culture experiments.

Conclusion: This study shows, for the first time to our knowledge, that consumption of cocoa flavanols can significantly affect the growth of select gut microflora in humans, which suggests the potential prebiotic benefits associated with the dietary inclusion of flavanol-rich foods. This trial was registered at clinicaltrials.gov as NCT01091922. *Am J Clin Nutr* 2011;93:62–72.

INTRODUCTION

Diet is a major lifestyle factor that can greatly influence the incidence and progression of chronic diseases such as cancer, cardiovascular disease, and diabetes (1–4). In terms of human intake, monomeric and oligomeric (procyanidins) flavanols are predominantly observed in fruit, vegetables, tea, red wine (5), and cocoa and cocoa products (6). Human dietary interventions with flavanol-containing cocoa indicated beneficial effects of flavanols on LDL oxidation (7), platelet aggregation (8), insulin sensitivity (9), and endothelial function (10–13). Because the flavanol monomer (–)-epicatechin and the dimeric procyanidin dimer B2 have shown bioavailability from cocoa in humans (12, 14), these

are thought to directly mediate observed cardiovascular-related benefits (10, 12). However, the majority of procyanidins in cocoa (6, 15) are not absorbed intact in the small intestine (16) and, instead, pass to the large intestine where they may influence the profuse and active microbiota that exists there.

The microbiota can be categorized as being either beneficial or potentially pathogenic because of its metabolic activities and fermentation end products (17). Health-promoting effects of the microflora may include immunostimulation, vitamin synthesis, and inhibition of pathogen growth, whereas detrimental effects include carcinogen production, diarrhea and constipation, and intestinal infections (17). There has been considerable interest in the dietary modulation of the microbiota, particularly via the use of probiotics and prebiotics (18, 19). Effective prebiotics have low digestibility and bioavailability, are selective for the growth and metabolism of commensal bacteria, and alter the microbiota to a healthy composition (20). Currently, the most widely used prebiotics are fructooligosaccharides and galactooligosaccharides, which enhance the growth of bifidobacteria and positively influence mineral absorption, lipid metabolism, and innate immunity (21). It has been proposed that an imbalance between the 2 dominant phyla of bacteria, the Bacteroidetes and the Firmicutes, plays a role in the progression of metabolic disorders such as obesity and diabetes (22, 23), whereas bacterial lipopolysaccharides may control the tone of the innate immune system and, thus, regulate the general inflammatory status, insulin resistance, and adipose tissue plasticity (22, 24).

We have previously shown that the flavanol monomer (+)-catechin significantly increases the growth of the *Clostridium coccoides*–*Eubacterium rectale* group, *Bifidobacterium* spp., and *Escherichia coli*, and significantly inhibits the growth of the *Clostridium histolyticum* group (25). Furthermore, tea phenolics

¹ From the Department of Food and Nutritional Sciences, The University of Reading, Reading, United Kingdom (XT, AR-M, JV, GRG, and JPES), and the Analytical and Applied Sciences Group, Mars Inc, Hackettstown, NJ (CK-U).

² Supported by a grant from the Reading University Endowment Trust Fund. Mars Inc (Hackettstown, NJ) donated the intervention test materials for the human study and in vitro work.

³ Address correspondence to JPE Spencer, Molecular Nutrition Group, School of Chemistry, Food and Pharmacy, University of Reading, Reading RG2 6AP, United Kingdom. E-mail: j.p.e.spencer@rdg.ac.uk.

Received July 21, 2010. Accepted for publication October 13, 2010.

First published online November 10, 2010; doi: 10.3945/ajcn.110.000075.

have been shown to affect the growth of pathogenic bacteria such as *Clostridium perfringens*, *Clostridium difficile*, and *Bacteroides* spp. more than do commensal anaerobes such as *Bifidobacterium* spp. and *Lactobacillus* spp. (26). These data suggest that the consumption of flavanol-rich foods may have the potential to support gut health through their ability to exert prebiotic-like activity. The aim of the current study was to validate the potential prebiotic potential of flavanol-containing foods in humans by using a randomized, double-blind, controlled, crossover, human trial.

SUBJECTS AND METHODS

Materials

Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich Co Ltd (Poole, United Kingdom) or Fisher (Loughborough, United Kingdom), and bacteriologic growth-media supplements were obtained from Oxoid Ltd (Basingstoke, United Kingdom). (+)-Catechin (>99% pure and (-)-epicatechin (>90% pure) were purchased from Sigma-Aldrich Co Ltd and were of HPLC grade. The cocoa extract used in the batch culture experiments (supplied by Mars Inc, Hackettstown, NJ) was an acetone-based, cocoa flavanol-enriched extract and characterized as follows (wt:wt): epicatechin (8%), catechin (1%), dimers and decamers (31% and 9%, respectively), theobromine (8%), caffeine (2%), anthocyanins (1%), sugars and carbohydrates (excluding fiber) (28%), fiber (1%), fat (2%), protein (3%), ash (3%), and moisture (3%). HPLC columns were purchased from Waters Co (Watford, United Kingdom) and Sumika Chemical Analysis Service (Singapore). Isopore (0.2 μm) membrane filters were obtained from Millipore Corp (Watford, United Kingdom). All oligonucleotide probes used for fluorescence in situ hybridization (FISH) were commercially synthesized and labeled with the fluorescent dye Cy3 (MWG-Biotech Ltd, Milton Keynes, United Kingdom). Sterilization of media and instruments was achieved by autoclaving at 121°C for 15 min.

Flavanol-containing test materials

Intervention drinks were provided by Mars Inc (Hackettstown, NJ) as dry, dairy-based cocoa-beverage mixes. All cocoa drinks were standardized for their total cocoa flavanol contents and profiles and closely matched for equal macro- and micronutrient contents, caloric loads, and theobromine (HCF: 185 mg theobromine; LCF: 177 mg theobromine) and caffeine (HCF: 15 mg caffeine; LCF: 17 mg caffeine) concentrations (Table 1). All cocoa drinks were similar in taste and supplied in individual sachets labeled with an anonymous 3-digit code. The cocoa drinks contained either 29 mg [low-cocoa flavanol (LCF) drink] or 494 mg (high-cocoa flavanol (HCF) drink) total cocoa flavanols (Table 1). The amount of total cocoa flavanols referenced here was defined as the sum of all monomeric flavanols and their oligomeric derivatives (dimers to decamers; ie, 2–10 monomeric subunits). Participants were instructed to mix the content of one cocoa sachet in \approx 150 mL water once daily and to consume the drink at the same time each day (\pm 1 h) after their evening meal. Drinks were dispensed as a full 4-wk allotment, and compliance was monitored by collection of used packets.

TABLE 1
Composition of reconstituted cocoa intervention drinks¹

Component	High-cocoa flavanol drink	Low-cocoa flavanol drink
Energy (calories)	113	112
Fat (g)	1.3	1.3
Saturated fat (g)	0.8	0.8
Cholesterol (mg)	4.8	4.6
Protein (g)	8.7	8.9
Carbohydrates (g)	16.3	15.9
Sugars (g)	9.6	9.0
Sodium (mg)	197	204
Potassium (mg)	507	573
Cocoa flavanols (total monomers to decamers) (mg)	494	29
Monomers (mg)	110	6
Epicatechin	89	3
Catechin	21	3
Dimers (mg)	99	11
Total trimers to decamers (mg)	285	12
Caffeine (mg)	15	17
Theobromine (mg)	185	177

¹ Volunteers were requested to consume one cocoa drink daily after their evening meal for a period of 4 wk.

Intervention study subjects

Twenty-two healthy volunteers (12 men, 10 women; mean \pm SD age: 30.2 \pm 11.8 y) with a body mass index (BMI; in kg/m²) between 20.2 and 25.4 (mean \pm SD BMI: 23.2 \pm 2.5) were recruited from the University of Reading and surrounding area. All female volunteers were self-reported premenopausal (stable and regular menstruation cycles). Volunteers were assessed before the start of the trial for good health and were selected according to certain exclusion and inclusion criteria. Inclusion criteria for subject participation in the study were as follows: provided a signed consent form, had an inclusive age of 18–50 y, were in good general health and not pregnant or lactating, and had an absence of an allergy to milk products, sensitivity to alkaloids or caffeine, gastrointestinal disorders (eg, chronic constipation, diarrhea, inflammatory bowel disease, irritable bowel syndrome, or other chronic gastrointestinal complaints), diabetes, hypertension (>140/90 mm Hg), anemia, and gall bladder problems. Volunteers who took probiotics (in any form), prebiotics, synbiotics, antibiotics, or antiinflammatory or blood lowering medication in a \leq 2-mo period before the study and smokers were also excluded. Volunteers were instructed not to consume such products during the study and not to alter their usual dietary or fluid intakes. Subjects selected for the study were asked to refrain from the following for 2 wk before and during the study (including the washout period): consumption of flavanol-rich foods including cocoa, chocolate, apples, and red wine, participation in vigorous exercise ($>3 \times 20$ min exercise/wk), and consumption of >120 g (women) and >168 g (men) alcohol (any form)/wk. No further dietary restrictions or recommendations were imposed, although participants were requested not to change their daily intakes of tea (green or black) or coffee. All subjects were healthy as assessed by their responses to a standard medical questionnaire and blood results (ie, normal liver enzyme, hemoglobin, hematocrit, and leukocyte counts) and an absence of glucose and protein in the urine. Written informed

consent was obtained from all subjects before their participation in the study. This study was conducted from February 2008 to October 2008.

Study design

The study was conducted according to the guidelines of the Declaration of Helsinki, and all procedures involving human subjects were approved by the University of Reading Research Ethics Committee (reference 06/37). The study was designed as a randomized, double-blind, crossover, controlled intervention trial in which volunteers were asked to consume either an HCF drink (494 mg) or an LCF drink (29 mg). Subjects were randomly assigned via restricted randomization to either the low- or high-flavanol group and asked to consume one cocoa beverage per day for 4 wk. This was followed by a 4-wk washout period before subjects switched to the alternate arm of the study. Women began each intervention arm at a similar stage in their menstrual cycle (follicular phase). Subjects were required to provide a fecal sample and blood and urine samples before, and at the end of, each intervention arm. Fecal and blood samples were collected in the fasted state, and 24-h urine samples were collected in vessels that contained ascorbic acid (1 mmol ascorbic acid/L) and HCl (0.01 M). Anthropometric measurements and systolic and diastolic blood pressure (BP) (Omron MX2 automatic digital upper-arm BP monitor; Milton Keynes, United Kingdom) were measured by a research nurse before and after each intervention period. BP measurements were recorded after subjects rested for 5 min in a seated position. Subjects were advised not to alter their usual calorie intakes and were asked to document any unusual symptoms or side effects and to keep a diary of illnesses and medications. All subjects completed a 4-d diet diary of their habitual dietary intakes during week 3 of each trial arm and during the week immediately preceding each study arm (ie, 1 wk prestudy and week 4 of the washout period). Food-intake data were analyzed with Dietplan 6 Pro software (Forestfield Software Ltd, Horsham, United Kingdom).

Fecal samples were collected in 1.0-L stool-collection pots, and 10 g fecal material was immediately mixed with 10 mL phosphate-buffered saline (PBS) (0.1M), and samples were stored at -80°C for the assessment of fecal water genotoxicity. The remainder of the sample was diluted with anaerobic phosphate buffer (0.1 M; pH 7.4) and homogenized in a stomacher for 2 min to obtain a fecal slurry. A total of 10 mL fecal slurry was vortexed for 1 min with glass beads and centrifuged at $1500 \times g$ for 15 min, and the supernatant fluid (2 mL) was further centrifuged for 5 min at $10,000 \times g$. Before FISH, duplicate samples were fixed overnight at 4°C with paraformaldehyde (4% wt:vol), and the remaining supernatant fluid was stored at -80°C for HPLC analysis. Plasma samples were drawn into vials containing EDTA, supplemented with ascorbate (1 mg ascorbate/mL), snap-frozen in liquid nitrogen, and stored at -80°C .

Fecal bacteria enumeration

To assess differences in bacterial populations, FISH was used with oligonucleotide probes designed to target specific diagnostic regions of 16S ribosomal RNA. The probes were commercially synthesized and labeled with the fluorescent dye Cy3. The bacterial groups studied for enumeration were *Bifidobacterium*

spp., *Bacteroides* spp., *Lactobacillus* and *Enterococcus* spp., *C. coccoides*–*E. rectale* group, *C. histolyticum* group, and *E. coli* by using the specific oligonucleotide probes Bif164 (5'-CAT CCG GCA TTA CCA CCC-3'), Bac303 (5'-CCA ATG TGG GGG ACC TT-3'), Lab158 (5'-GGT ATT AGC A(T/C)C TGT TTC CA-3'), Erec482 (5'-GCT TCT TAG TCA GGT ACC G-3'), His150 (5'-TTA TGC GGT ATT AAT CT(T/C) CCT TT-3'), and Ec1531 (5'-CAC CGT AGT GCC TCG TCA TCA-3'), respectively. For total bacterial counts, a 4, 6-diami-dino-2-phenylindole nucleic acid stain was used. Fixed samples (in 4% paraformaldehyde; 24 h; 4°C) were centrifuged at $1500 \times g$ for 5 min, washed twice with PBS (0.1 M; pH 7.0), resuspended in a mixture of PBS and 99% ethanol (1:1 vol:vol) and stored at -20°C for 1 h. This process was used for all samples except for samples probed with Lab158. Samples probed with Lab158 were subjected to an additional enzyme step (25 mmol tris-HCl/L, 10 mmol EDTA/L, 585 mmol sucrose/L, 5 mmol CaCl_2 /L, 0.3 mg sodium taurocholate/mL, 2 mg lysozyme/mL, and 0.1 mg lipase/mL) for 60 min at 37°C to increase cell permeability.

Cell suspensions were added to the filtered sterilized hybridization mixture (30 mmol tris-HCl/L, 1.36 M NaCl, and 0.15% sodium dodecyl sulfate; pH 7.2) and were left overnight to hybridize at the appropriate temperature for each probe. Hybridized mixtures were vacuum filtered with isopore-membrane filters with a 0.2- μm pore size (Millipore Corp), and filters were placed onto labeled glass slides. A drop of Slowfade (Molecular Probes, Egan, OR) was added to each slide, a cover slip was placed on top of each filter, and slides were stored in the dark at 4°C . Slides were examined after 60 min with a fluorescent microscope (Nikon Eclipse E400; Nikon, Surrey, United Kingdom). 4, 6-Diami-dino-2-phenylindole stained cells were examined under ultraviolet light, and a DM510 light filter was used to count microorganisms hybridized with the probes. For each slide, 15 random different fields of view were counted. Numbers of bacteria were calculated by using the following formula:

$$\text{Number of bacteria} = \log_{10}[15.56 \times A1 \times 14,873.74 \times (1000/B1)] \quad (1)$$

where 15.56 is the dilution factor, A1 is the average count from 15 fields of view, 14,873.74 is the area of the field of view, and B1 is the sample volume expressed in microliters. The number of bacterial cells was expressed per milliliter of culture. To determine changes in the bacterial population between treatments we used an index for specific bacteria (Isb), calculated as follows:

$$\text{Isb} = [\text{Ns}(T1) - \text{Ns}(T0)] - [\text{Nc}(T1) - \text{Nc}(T0)] \quad (2)$$

where Ns is the number \log_{10} of specific bacteria in a specific test sample (ie, high-flavanol intervention), Nc is the number \log_{10} of specific bacteria in the control (ie, low-flavanol intervention), T1 is a specific time point (ie, end of the intervention arm), and T0 denotes 0 h or the start of the intervention arm. For batch cultures, the time point used was 6 h.

Fecal water genotoxicity

Fecal water genotoxicity was assessed by using single cell gel electrophoresis (27). Fecal water was obtained from fecal samples

(1 g fecal sample/mL) of subjects on the intervention trial, and assessment of its genotoxicity was measured as previously described (28). For quantification, 100 cells were scored for DNA intensity in the head and tail region with Komet 5 software (Andor Technologies, Nottingham, United Kingdom). All samples were performed in triplicate, and slides were processed in ≤ 24 h.

Flavanol quantification

Plasma samples were isolated from fresh blood, mixed with ascorbic acid (1 mg ascorbic acid/mL), and stored at -80°C until analysis. Fecal water was obtained by centrifugation ($35,000 \times g$; 2 h; 4°C) of fecal samples (1 g fecal sample/mL) and filtration of the supernatant fluid ($0.2 \mu\text{m}$). These were stored at -80°C until the time of analysis. Urine samples also underwent $0.2 \mu\text{m}$ filtration and were stored at -80°C until analysis. All samples were subjected to β -glucuronidase and sulfatase treatment, extracted, concentrated, and analyzed for flavanol concentrations with a Hewlett-Packard 1100 series chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a fluorescent detector as previously detailed (12). The concentration of each compound was measured by using an external calibration curve produced with the use of authentic standards. Total flavanol concentrations [degree of polymerization (DP) 1–10] in fecal water and batch culture supernatant fluids were analyzed as previously described (15). Concentrations of flavanols and oligomers present in batch culture samples were expressed as the percentage of their total amount present at T0 (ie, before incubation with bacteria). Because no flavanols were detectable in noninoculated human fecal samples, quantification was not undertaken. For the analysis of catechin, epicatechin, procyanidin dimer B2, caffeine and theobromine, batch culture supernatant fluids were further subjected to reversed-phase analysis with a C18 Nova-Pak column (4.6×250 mm; $4 \mu\text{m}$) (Waters Co) as previously reported (25), and eluants were monitored by photodiode array detection at 280 nm to detect caffeine, theobromine, flavanols, and phenolic acid metabolites, and spectra of analytes were monitored over the 220–600-nm range. Concentrations of epicatechin, catechin, dimer B2, caffeine, and theobromine were measured via construction of external calibration curves by using authentic standards.

Biochemical analyses

The blood samples collected in lithium and heparin tubes were centrifuged ($1700 \times g$; 10 min; 4°C) immediately after collection. Samples were also collected in serum separation tubes and allowed to stand for 30 min before centrifugation ($1300 \times g$; 10 min; 21°C). All samples were aliquoted and frozen at -80°C until analysis. Plasma concentrations of total cholesterol, LDL cholesterol, HDL cholesterol, glucose and triacylglycerol (TAG) were assayed on an ILAB 600 chemistry analyzer (Instrumentation Laboratory, Warrington, United Kingdom) by using enzyme-based colorimetric tests supplied by Instrumentation Laboratory. The following variables were measured in all samples: total cholesterol, LDL cholesterol, HDL cholesterol, glucose, triacylglycerol, and C-reactive protein (ultrasensitive) (CRP) concentrations. The total antioxidant capacity of fecal water was also assessed with the ILAB 600 chemistry analyzer (Instrumentation Laboratory) with a kit supplied by Medicon SA (Gerakas, Greece) and expressed as millimoles of antioxidant per liter of Trolox (Sigma, Poole, United Kingdom).

Inoculation of fecal microflora with cocoa extract

Fecal samples were collected from 3 separate individuals. All volunteers were in good health and had not ingested antibiotics for ≥ 6 mo before the study. Mixed-bacteria batch culture vessels were prepared and performed as previously described (25). Vessels were inoculated with 15 mL fecal slurry (1:10 wt:vol), and batch cultures were run under anaerobic conditions for a period of 24 h before the addition of the high-flavanol cocoa powder extract (1 mg extract/mL; ≈ 0.4 mg/mL flavanols). The concentration of flavanols was selected to reflect the approximate gastrointestinal concentrations of flavanols achieved after the high-flavanol intervention in the human study [450 mg flavanols/d; with the assumption of a stomach volume of 1–1.5 L, this equated to 0.3–0.45 mg total flavanols/mL]. Samples (3 mL) were collected at 4 time points (0, 2, 4, and 6 h) for flavanol analysis (DP 1–10, monomers and dimers) by HPLC and at 6 h for bacterial enumeration. Supernatant fluids were obtained by centrifugation ($35,000 \times g$; 2 h; 4°C) of batch culture samples, filtration ($0.2 \mu\text{m}$), and storage at -80°C . For the flavanol analysis, ascorbic acid (1 mg ascorbic acid/mL) was added to supernatant fluid before freezing. Control experiments were also run in which the extract in basal medium without fecal slurry inoculations was incubated.

Statistical analyses

All statistical analyses of data occurred in advance of the authors being unblinded. For bacterial enumeration work, changes in bacterial groups, which were calculated by using the index of specific bacteria, were expressed as mean values and plotted with SDs. All data presented in the text and figures are means \pm SDs. Within each treatment group (ie, LCF or HCF), changes in bacterial, physiologic, and biochemical variables from baseline values were analyzed by paired Student's *t* test. For multiple comparisons, data were analyzed by using a 2-factor repeated-measures analysis of variance with time and treatment as the 2 factors. Post hoc analyses were performed by using the Tukey-Kramer test. All statistical analyses were performed with SPSS version 12.1 software (SPSS Inc, Chicago, IL). All data were checked for normality and log transformed when necessary before statistical analyses. Univariate correlations were calculated by using Pearson's *r*. A multivariate regression analysis was performed to identify individual bacteria as independent predictors for BP and CRP. Statistical significance was assumed if a null hypothesis could be rejected at $P = 0.05$.

RESULTS

Subject compliance and food intake

Two subjects withdrew from the study for the following reasons: one subject started antibiotic treatment and another had problems providing fecal samples. The drinks were well tolerated by all subjects who completed the study, and no intolerances or adverse events were reported. Volunteer compliance was assessed by return of used and unused cocoa sachets and by self-reported cocoa-beverage intake (by means of a daily diet diary), which indicated that compliance was $>95\%$. There were no significant differences in energy intakes or macronutrient intakes, including protein, fat, carbohydrate, or alcohol intakes, across treatments



or compared with those at baseline (Table 2). The dietary fiber intake of participants was relatively high (mean: 24.4 g dietary fiber/d), which reflected an above-average intake of fruit and vegetables (319 g fruit and vegetables/d) by the volunteers. Although each intervention drink delivered 3–4 g fiber/d, there was no significant change in the amount of dietary fiber consumption measured between baseline and completion of either intervention arm. In accordance with the energy-intake data, there was no significant change in BMI throughout the intervention period. No significant changes in tea (green or black) or coffee consumption were observed in any of the individuals.

Physiologic and biochemical variables

BP measurements were in the healthy range, and no significant change in either systolic or diastolic BP (in mm Hg) after the consumption of either intervention drink was recorded, despite a -4.3 -mm Hg reduction in systolic BP after the HCF intervention. Flavanol intake had no effect on plasma concentrations of LDL-cholesterol, HDL-cholesterol, and glucose ($P > 0.05$). However, a significant reduction in total cholesterol concentrations was observed after intervention with either the HCF or LCF drinks ($P = 0.03$ and 0.02 , respectively) (Table 3), although there was no difference between intervention groups ($P > 0.05$). Plasma triacylglycerol concentrations were not significantly affected by the LCF intervention but were significantly reduced after the 4-wk intervention with the HCF beverage with respect to baseline concentrations ($P = 0.04$) and those of the LCF control ($P = 0.02$) (Table 3). A significant reduction in plasma CRP concentrations was also observed after the 4-wk HCF intervention ($P < 0.05$), although this reduction occurred from a low baseline, which was predicted in our healthy study population, and, thus, may not represent a change of clinical significance. No significant differences were measured after the LCF intervention (Table 3).

Effect of cocoa flavanol intake on human fecal microflora

Changes in bacterial population amounts were assessed in fecal samples of all volunteers before and after each intervention arm and were presented as the \log_{10} change from the respective

baseline measures (Figure 1). There were no significant differences in the number of total bacteria, *Bacteroides* spp., or *E. coli* after intake of either the HCF or LCF drinks for 4 wk ($P > 0.05$) (Figure 1), which indicated that flavanol consumption had no effect on the growth of these bacteria as a function of time or treatment. However, there was a significant difference in the population amounts of *Bifidobacterium* spp., the *C. histolyticum* group, *E. rectale*–*C. coccoides* group, and *Lactobacillus* and *Enterococcus* spp. in the human fecal samples of subjects in response to both interventions. The intervention with HCF for 4 wk led to a significant increase in the numbers of *Lactobacillus* and *Enterococcus* spp. ($P = 0.007$). This increase in numbers of *Lactobacillus* and *Enterococcus* spp. in response to HCF consumption was significantly greater than that observed after the consumption of the LCF drink ($P < 0.001$). Daily HCF consumption also resulted in a significantly larger increase in the number of *Bifidobacterium* spp. in fecal samples collected postintervention (Figure 1), whereas no significant increase in the number of *Bifidobacterium* spp. was measured after the daily consumption of the LCF drink. Interventions with HCF and LCF beverages for 4 wk led to a significant increase in numbers of the *E. rectale*–*C. coccoides* group, although there were no significant differences between the 2 groups. HCF consumption for 4 wk led to a significant decrease in the fecal numbers of the *C. histolyticum* group ($P = 0.042$), whereas LCF intake resulted in a significant increase in the fecal numbers of the *C. histolyticum* group (Figure 1). There was a significant difference in fecal amounts of the *C. histolyticum* group between the 2 intervention groups ($P < 0.001$). There were no significant differences between the 2 baseline measurements of any of the bacteria ($P > 0.05$), which indicated that the washout period was effective.

Bacterial changes predicted changes in CRP concentrations but not in other biochemical markers or BP

In addition to the mean reduction in CRP concentrations after the HCF intervention (Table 3), we also observed significant univariate correlations between changes in the amounts of specific bacteria and plasma CRP concentrations (bifidobacteria: $r =$

TABLE 2

Calculated daily nutrient intakes at baseline (Pre) and week 4 (Post) of low- and high-cocoa flavanol intervention arms ($n = 20$)¹

	Low-flavanol cocoa		High-flavanol cocoa	
	Pre	Post	Pre	Post
Energy (kJ/d)	8398 ± 334	8518 ± 410	8230 ± 439	8361 ± 386
Protein (g/d)	72 ± 5	73 ± 3	71 ± 4	72 ± 4
Fat (g/d)	73 ± 1	75 ± 6	69 ± 5	72 ± 5
SFA (g/d)	26 ± 8	27 ± 3	24 ± 3	26 ± 4
PUFA (g/d)	11 ± 1	12 ± 2	10 ± 1	11 ± 1
MUFA (g/d)	25 ± 5	28 ± 3	25 ± 2	27 ± 3
Carbohydrate (g/d)	233 ± 16	242 ± 16	226 ± 16	231 ± 16
Total dietary fiber (g/d)	23.8 ± 7.2	24.1 ± 2.8	22.9 ± 2.7	23.3 ± 2.6
Dietary cholesterol (mg/d)	245 ± 31	259 ± 30	265 ± 36	244 ± 30
Alcohol (g/d)	10 ± 3	11 ± 4	11 ± 3	10 ± 3

¹ All values are means ± SDs. SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid. There were no significant differences between dietary intake of energy or macronutrients between baseline measures and either the high-cocoa flavanol or low-cocoa flavanol intervention arms at week 4 ($P > 0.05$). Significance was calculated by the Tukey-Kramer test after 2-factor repeated-measures ANOVA with time and treatment as the 2 factors.

TABLE 3

Anthropometric and biochemical variables before (Pre) and after (Post) the 4-wk intervention with either the low-cocoa flavanol or high-cocoa flavanol drink ($n = 20$)¹

	Low-flavanol cocoa		High-flavanol cocoa	
	Pre	Post	Pre	Post
BMI (kg/m ²)	23.1 ± 2.02	23.2 ± 2.06	23.2 ± 2.1	23.2 ± 2.1
Diastolic BP (mm Hg)	71.4 ± 12.95	72.0 ± 11.75	69.8 ± 6.9	68.8 ± 7.2
Systolic BP (mm Hg)	107.3 ± 7.81	105.8 ± 11.32	110.0 ± 11.6	105.7 ± 12.8
Total cholesterol (mmol/L)	4.70 ± 0.19	4.31 ± 0.1 ²	4.77 ± 0.2	4.34 ± 0.2 ²
HDL cholesterol (mmol/L)	1.36 ± 0.08	1.29 ± 0.08	1.39 ± 0.09	1.33 ± 0.11
LDL cholesterol (mmol/L)	2.63 ± 0.16	2.50 ± 0.14	2.57 ± 0.17	2.48 ± 0.16
Triacylglycerol (mmol/L)	1.06 ± 0.08	1.05 ± 0.07	1.07 ± 0.08	0.87 ± 0.09 ^{2,3}
Glucose (mmol/L)	5.13 ± 0.10	5.08 ± 0.10	5.13 ± 0.13	5.18 ± 0.12
C-reactive protein (mg/mL)	0.26 ± 0.11	0.31 ± 0.14	0.27 ± 0.12	0.19 ± 0.09 ^{2,3}
Fecal water TAC (mmol/L Trolox ⁴)	479.2 ± 48.3	459.7 ± 41.3	487.2 ± 46.6	473.2 ± 55.6

¹ All values are means ± SDs. BP, blood pressure; TAC, total antioxidant capacity. Significance was calculated by the Tukey-Kramer test after 2-factor repeated-measures ANOVA with time and treatment as the 2 factors.

² Significantly different from baseline, $P < 0.05$

³ Significantly different from low-cocoa flavanol interventions, $P < 0.01$.

⁴ Trolox (Sigma Chemical Co, Poole, United Kingdom).

–0.438, $P < 0.05$; lactobacilli: $r = -0.492$, $P < 0.01$). Significance was not observed for clostridia or any of the other bacteria measured. In a multivariate regression analysis that included all bacteria, only lactobacilli predicted the magnitude of CRP reductions ($P < 0.05$, $R^2 = -0.33$ for the model), which suggested that this bacterial group may have been, at least in part, involved in mediating the observed reduction in plasma CRP concentrations. No correlations were shown between CRP concentrations and any of the other data, including BP.

Flavanol absorption and metabolism

No flavanols were detected in the fecal water of any of the volunteers, either before or after intervention with the HCF or LCF beverages, which suggested that all nonabsorbed flavanols were extensively catabolized. Fasting (no food, drink, or flavanol intervention for 12 h) plasma concentrations of epicatechin were shown to significantly increase from 24.7 ± 6.6 to 72.9 ± 12.7 nmol/L after the daily consumption of the HCF beverage ($P < 0.01$). In contrast, fasted epicatechin concentrations did not significantly change after the consumption of the low-flavanol beverage (Figure 2A). Concentrations of catechin and 3'- and 4'-*O*-methyl-epicatechin were undetectable in fasted plasma samples. However, epicatechin, 3'- and 4'-*O*-methyl-epicatechin were detectable in 24-h urine samples and were shown to increase after the HCF intervention (Figure 2B). Low concentrations of flavanols were detected in urine at baseline, but no increases were noted after the daily consumption of the low flavanol beverage for 4 wk (Figure 2B). No catechin or 3'- and 4'-*O*-methyl catechin was detected in urine samples.

Effect on fecal water genotoxicity and antioxidant status

Measurements of fecal water genotoxicity were calculated by assessment of the potential of fecal water to induce DNA-strand breakage in the comet assay. The intervention for 4 wk with the HCF beverage resulted in fecal water samples that showed significantly less genotoxic potential relative to that measured at baseline (baseline: $86.5 \pm 1.4\%$ intact DNA; high flavanol co-

cocoa: 89.9 ± 0.9 intact DNA; $P < 0.05$). In contrast, the intervention with LCF drinks did not significantly alter the genotoxic potential of fecal water (baseline: $87.1 \pm 1.4\%$ intact DNA; high flavanol cocoa: 88.3 ± 1.1 intact DNA). This was

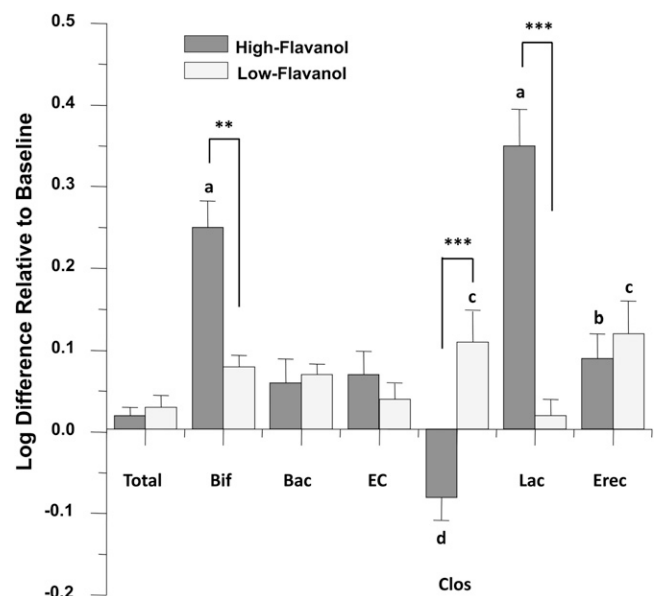


FIGURE 1. Mean (\pm SD) log₁₀ bacterial numbers per gram of feces as measured by fluorescence in situ hybridization showing the effect of high- and low-cocoa flavanol cocoa consumption on fecal bacterial numbers in the human large intestine ($n = 20$). Values indicate log₁₀ changes after 4 wk of intervention with either the high- or low-flavanol cocoa relative to preintervention amounts. Data were analyzed by using 2-factor repeated-measures ANOVA with time and treatment as the 2 factors. Post hoc analyses were performed by using the Tukey-Kramer test. ^{a,b,c}Significant increases in fecal bacteria numbers between baseline and 4 wk of intervention: ^a $P < 0.001$, ^b $P < 0.01$, ^c $P < 0.05$. ^dSignificant decreases in fecal bacteria numbers between baseline and 4 wk of intervention, $P < 0.01$. ^{***}Significant differences in bacterial numbers between high- and low-flavanol interventions: ^{**} $P < 0.001$, ^{***} $P < 0.001$. Bif, *Bifidobacterium* spp.; Bac, *Bacteroides* spp.; EC, *Escherichia coli*; Clos, *Clostridium* *holyticum* group; Lac, *Lactobacillus* and *Enterococcus* spp.; Erec, *Eubacterium rectale*–*Clostridium coccoides* group.

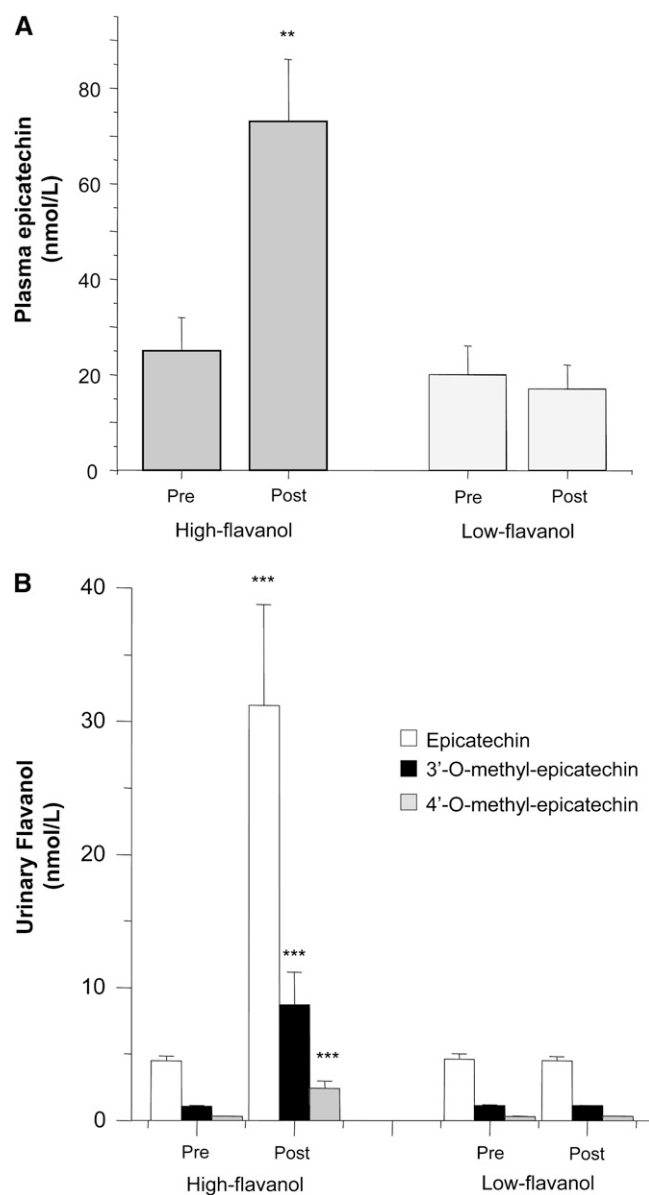


FIGURE 2. Mean (\pm SD) absorption and excretion of flavanols after low- or high-cocoa flavanol consumption ($n = 20$). A: Plasma concentrations of (–)-epicatechin before (Pre) and after (Post) 4 wk of intervention. Within each treatment group (ie, low- or high-cocoa flavanol groups), changes in flavanol concentrations from baseline values were analyzed by paired Student's t test. B: Urinary concentrations of (–)-epicatechin, 3'- O -methyl-epicatechin, and 4'- O -methyl-epicatechin detected in 24-h urine samples. ****Significant difference between baseline and after intervention: ** $P < 0.01$, *** $P < 0.001$.

also reflected by a significant difference between the 2 intervention groups ($P = 0.031$). However, there were no significant differences in the total antioxidant activity of the fecal water between treatments or compared with that at baseline ($P > 0.05$) (Table 2).

Microflora changes induced by cocoa flavanols in a mixed-batch culture system

To support in vivo observations, we also investigated the effect of flavanol-enriched cocoa extract on fecal bacteria growth in an

anaerobic, pH-controlled, stirred batch culture–fermentation system. The incubation of the microflora with the flavanol-enriched cocoa extract (1 mg extract/mL; 6 h) led to a significant increase in the growth of *Lactobacillus* spp. ($P < 0.001$), a significantly greater rate of growth of *Bifidobacterium* spp. ($P < 0.05$), and a significant decrease in the growth of the *C. histolyticum* group ($P < 0.001$) (Figure 3). Upon incubation with the microflora, there was a rapid change in the total flavanol profile, with monomers, dimers, and oligomers (up to an octamer) greatly reduced after 6 h of incubation (Figure 4, A and B). Amounts of monomeric, dimeric, and higher flavanol oligomers (trimer to nonamer) all decreased markedly during incubation with the gut bacteria. The amounts of trimer were observed to significantly increase at 0.5 h; however, these increases were transient because they were no longer apparent after 2 h (Figure 4B).

A reversed-phase HPLC analysis confirmed the time-dependent loss of epicatechin, catechin, and dimer B2 upon exposure to the microflora (Figure 5). Concomitant with these reductions, there was the appearance of a new product, M1, which was confirmed by mass spectral analysis to be 5-(3',4'-dihydroxyphenyl)- γ -valerolactone (molecular weight of 207 detected in the negative-ion mode). The appearance of this metabolite agreed with previous observations in which pure epicatechin and catechin were exposed to fecal bacteria in a similar model (25). Amounts of theobromine and caffeine, observed naturally in cocoa, remained constant throughout the incubation period (Figure 5). In control experiments, no significant degradation of the flavanols (monomeric or oligomeric forms) was observed when they were added to batch culture systems without fecal inoculum (data not shown).

DISCUSSION

The prebiotic effects of fructooligosaccharides and galactooligosaccharides (29) are thought to underpin certain functional outcomes in the large gut, such as an ability to inhibit intestinal cancer progression, influence mineral absorption, effect lipid metabolism, and regulate innate immunity (21). However, there is limited information regarding the ability of other dietary components, including flavonoids, to influence the growth of selected intestinal bacteria (25, 30). To address this, we conducted the first human-intervention study, to our knowledge, designed to investigate the influence of high flavanol intake on the growth of the human fecal microbiota. We showed that a 4-wk, daily ingestion of an HCF beverage that contained 494 mg flavanols significantly increased the growth of *Lactobacillus* spp. and *Bifidobacterium* spp. relative to that of a control LCF beverage that contained only 29 mg flavanols. Because the intervention drinks were matched for fiber amounts and did not contain any recognized prebiotic materials, this suggests that these changes in bacterial growth were mediated by cocoa flavanols. This was supported by our in vitro mixed batch culture experiments that indicated that direct incubation of the fecal microflora with a flavanol-enriched cocoa extract that contained <1% overall fiber (ie 10 mg fiber/mL in batch culture) also induced a significant increase in the growth of *Lactobacillus* spp. and *Bifidobacterium* spp.

The increase in the growth of *Lactobacillus* spp. in response to cocoa flavanols is of note because this bacterial group is associated with beneficial effects in the gut, including an ability to



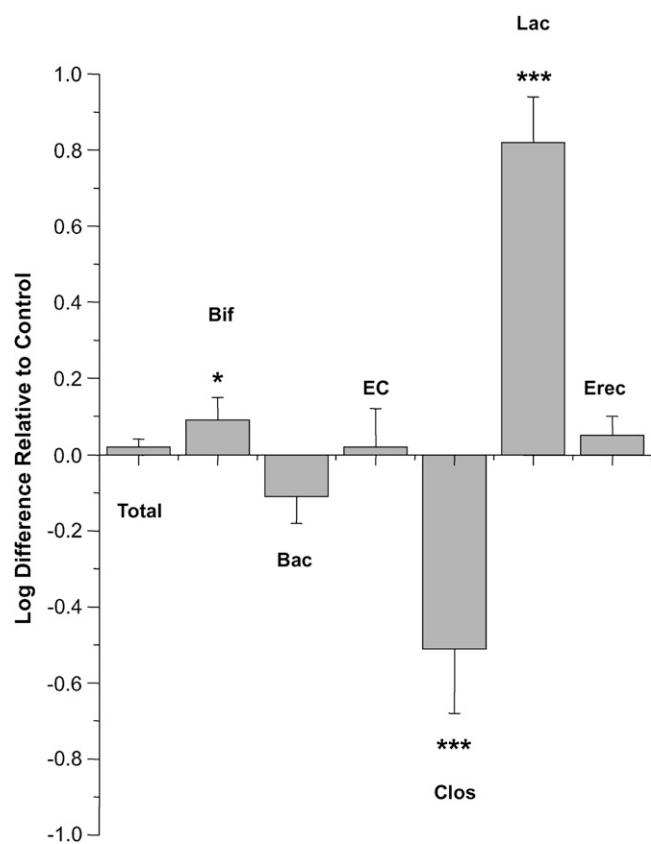


FIGURE 3. Mean (\pm SD) changes in bacterial populations during the fermentation of flavanol-enriched cocoa extract in a pH-controlled fecal batch culture showing the influence of cocoa flavanols on the growth of the colonic microflora ($n = 6$). Samples were collected at 6 h and analyzed by fluorescence in situ hybridization. Bacterial changes were calculated by using the index of specific bacteria and are expressed as \log_{10} cells per milliliter. Changes in bacteria from baseline values were analyzed by paired Student's t test. ****Significant changes in bacterial growth were calculated by comparing the number of a specific bacterial group in the treatment group to the number observed in a control group at the same time point: * $P < 0.05$, *** $P < 0.001$. Bif, *Bifidobacterium* spp.; Bac, *Bacteroides* spp.; EC, *Escherichia coli*; Clos, *Clostridium histolyticum* group; Lac, *Lactobacillus* and *Enterococcus* spp.; Erec, *Eubacterium rectale*-*Clostridium coccoides* group.

prevent the growth of pathogenic organisms (31), and most currently accepted prebiotics do not elicit changes in lactobacilli (18). The increased growth of bifidobacteria has been strongly associated with positive effects in the large intestine (29) through the ability of the bifidobacteria to inhibit the growth of pathogens, drive the synthesis of certain vitamins (eg, vitamin B-9) (17, 32), and reduce plasma cholesterol concentrations (33). In addition, both bacterial groups stimulate the production of beneficial organic acids such as lactate and acetate and inhibit the colonization of the gut epithelial layer by pathogenic bacteria (17). There is also an emergent interest in the potential cross-talk between specific probiotic bacteria and the host immune system (34, 35). In this context, we report that flavanol-induced increases in the growth of *Lactobacillus* spp. and *Bifidobacterium* spp. may have been partly responsible for the observed reductions in the plasma CRP concentration, which is a blood marker of inflammation and a hallmark of the acute-phase response (36, 37), although no causal relation between the 2 variables was inferred. The CRP concentration is a specific

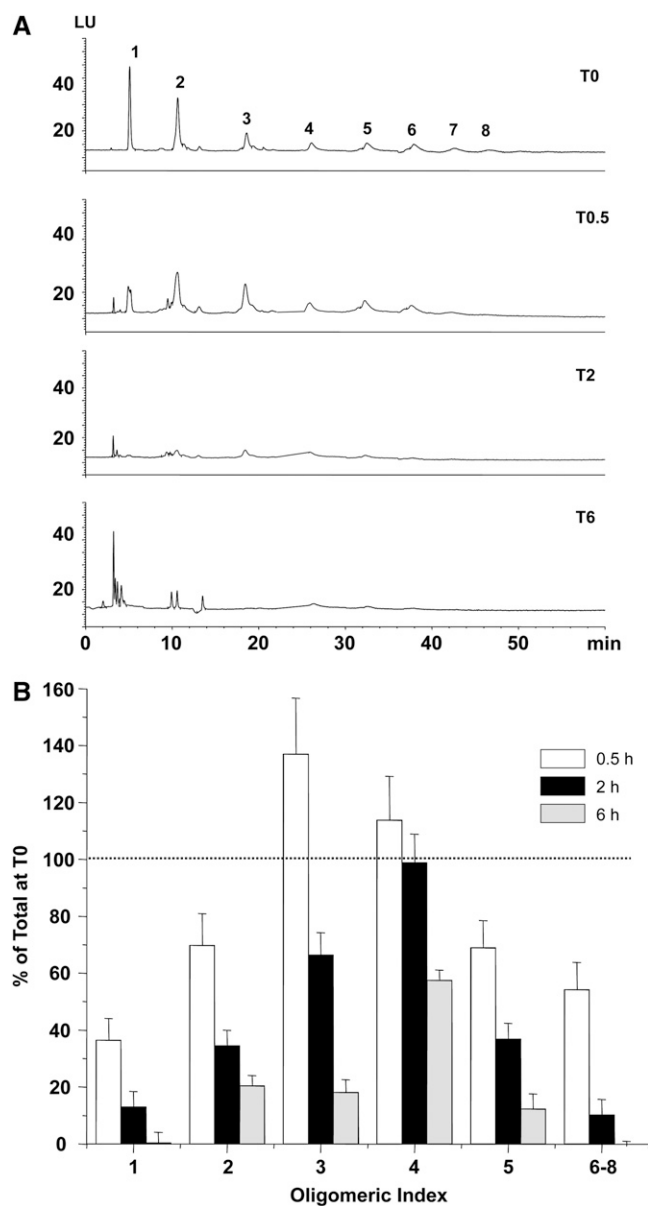


FIGURE 4. Bacterial-dependent metabolism of the flavanol-enriched cocoa extract. A: Typical HPLC chromatogram with fluorescent detection (excitation: 276 nm; emission: 316 nm) of fecal supernatants derived from the batch culture with the flavanol-enriched cocoa extract (1 mg extract/mL) collected at time (T) 0, 0.5, 2, and 6 h of incubation with human colonic bacteria. Numbers 1–8 indicate flavanol monomer to octamer, respectively. B: Percentage of the total flavanol oligomer remaining in the match culture system at 0.5, 2, and 6 h as measured by HPLC with fluorescence detection. LU, luminosity units.

predictor of cardiovascular event risk in healthy individuals (38), and its reduction in our study, albeit from a low baseline concentration, agree with other lines of evidence that link flavanol intake to cardiovascular benefits (12). In addition to changes in probiotic bacteria, the HCF intervention also induced a significant decrease in the *C. histolyticum* group, which suggested that cocoa-derived flavanols also have an inhibitory effect on the growth of this bacterial group, which includes *Clostridium perfringens*, which is a known pathogen implicated in the progression of colonic cancer and the onset of inflammatory bowel disease (39).

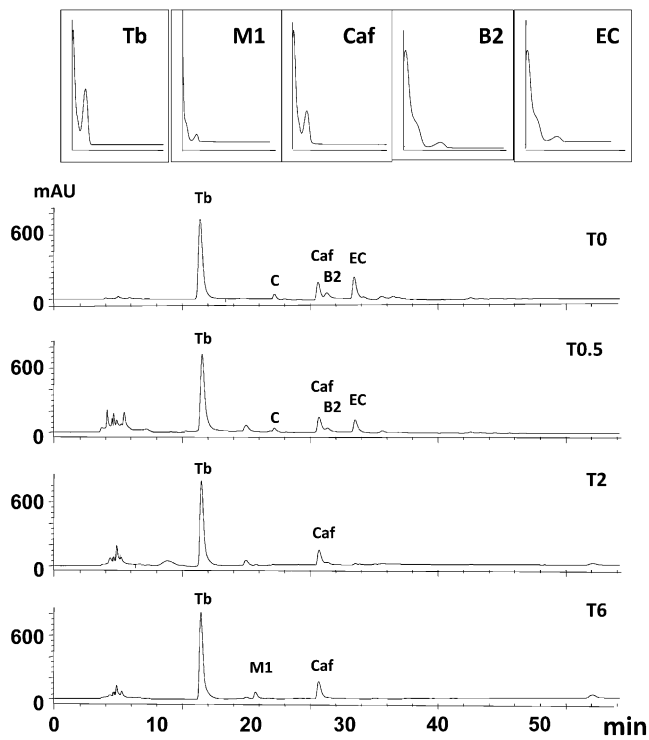


FIGURE 5. Bacteria-dependent metabolism of the flavanol-enriched cocoa extract. Typical HPLC chromatogram with photodiode array spectra (200–600 nm) of fecal supernatants derived from the batch culture with flavanol-enriched extract (1 g extract/L) collected at time (T) 0, 0.5, 2, and 6 h of incubation with human colonic bacteria. Tb, theobromine; M1, 5-(3',4'-dihydroxyphenyl)- γ -valerolactone; Caf, caffeine; B2, dimer B2; EC, epicatechin; C, catechin. mAU, milli-absorbance units.

Tzounis et al (25) reported that flavanol monomers induced a significant increase in the growth rate of *Bifidobacterium* spp. and a decrease in the growth rate of the *C. histolyticum* group in vitro. Although the results are consistent with those of our current study and other in vitro work (26), our current in vivo and in vitro data indicate that intervention with a natural mixture of flavanol monomers and oligomers causes an additional large increase in the growth of *Lactobacillus* spp. These differences may suggest that this bacterial group is preferentially able to catabolize flavanol oligomers and, thereby, use them as an energy source for growth. However, in contrast to the work of Tzounis et al (25) with monomeric flavanols (25), regular HCF intake did not lead to significant changes in the growth of the *C. coccoides*–*E. rectale* group, which indicated that oligomeric flavanols appeared to counteract the increase in the growth of this bacterial group. This was supported by our batch culture data with the flavanol-enriched cocoa extract, which also showed that, in the presence of this extract that contained both monomers and oligomers, there was no change in the growth of the *C. coccoides*–*E. rectale* group, despite significant changes in other bacterial populations.

Throughout the study, we recorded good compliance in both the HCF and LCF intervention arms, which was confirmed by the increased urinary flavanol excretion in the high- but not the low-flavanol arm of the study. Although fasted plasma concentrations of flavanols were small compared with those observed in acute-intervention studies (12), they were in agreement with previous

longer-term studies with cocoa flavanols (40). Although the 4.3-mm Hg decrease in systolic BP was shown not to be significant, previous chronic high-flavanol interventions have suggested a change of this magnitude may be clinically significant (–2.9 to –4.2 mm Hg) (13, 45). However, the regular intake of cocoa flavanols did significantly reduce plasma triglyceride concentrations in a similar manner to nondigested, fermentable carbohydrates, which regulate both lipemia and triglyceridemia (46–48). Because reductions in plasma triglyceride concentrations have not been consistently observed in human-intervention studies with flavanol-rich cocoa products (49–51), additional studies are needed to confirm these findings. In contrast to urine and plasma measurements, no flavanols were detectable in fecal samples, and there was no variation in the fecal water antioxidant capacity, which presumably reflected the relatively rapid catabolism of flavanols (6 h in our in vitro experiments) compared with the long transit time of material in the healthy human large intestine (18–72 h). It has been suggested that the vast majority of ingested proanthocyanidins are extensively metabolized to low-molecular-weight aromatic compounds (41–43). Despite this, HCF intake for 4 wk resulted in a significant decrease in the genotoxicity of fecal water, which may parallel previous observations linking flavanols with an ability to inhibit adenocarcinoma cell proliferation (44).

The fecal bacterial changes observed in our study are roughly comparable, in absolute terms, to those observed with established prebiotics, such as fructooligosaccharides and galactooligosaccharides. For example, fructooligosaccharides and galactooligosaccharides (10 mg fructooligosaccharides and galactooligosaccharides/mL) induce an increase of $\approx 1\text{-log}_{10}$ units in the numbers of *Bifidobacterium* spp. and/or *Lactobacillus* spp. (52, 53), whereas we showed that an enriched cocoa extract that contained 0.4 mg flavanols/mL was capable of inducing an $\approx 0.6\text{-log}_{10}$ increase in *Lactobacillus* spp. In human intervention studies, fructooligosaccharides and galactooligosaccharides (5 g fructooligosaccharides and galactooligosaccharides/4 wk) induced an increase in *Bifidobacterium* spp. and/or *Lactobacillus* spp. between 0.5- and 1- \log_{10} unit (54, 55). Although we recorded smaller increases in *Bifidobacterium* spp. ($\approx 0.2\text{-log}_{10}$ units) and *Lactobacillus* spp. ($\approx 0.35\text{ log}_{10}$ units), the amount of cocoa flavanols consumed daily was much lower (494 mg /d) than the amount used in carbohydrate studies (5 g/d). Although linear correlations between the consumption of dietary flavanols and changes in specific colonic bacteria may not exist, it seems likely that, in addition to carbohydrate prebiotics, flavanols, and perhaps most notably oligomeric flavanols, may also produce significant beneficial changes in the colonic bacteria.

In conclusion, we used a randomized, double-blind, crossover, controlled intervention study to show, for the first time to our knowledge, that the regular dietary inclusion of foods rich in flavanols (in this case, cocoa flavanols) could have significant effects on the growth of select gut microbiota in vivo. These in vivo findings coupled with the in vitro data suggest that flavanols themselves are mediators of these effects. Such changes in the growth of the microflora were seen in the context of no significant changes in diet, which supported the notion that the affects appeared to be driven by cocoa flavanols. The fact that these flavanol-induced changes were observed in the context of a balanced diet (ie, in line with American Heart Association healthy diet guidelines) underscores their potential to induce

specific effects in the gut and perhaps lends further credibility to the importance of phytonutrients and plant-based diets to overall health. Indeed, epidemiologic data has long supported that plant-based diets are strongly associated with improved gastrointestinal health. Although further research is required, the results of the current study suggest that the phytonutrient components of plants may be important contributors to these benefits.

The authors' responsibilities were as follows—XT: performed the majority of the experimental work and contributed to the study design; AR-M: coordinated the HPLC analysis and collaborated on the manuscript preparation; JV, GRG, and CK-U: contributed to the study design and manuscript preparation; JPES: contributed to the study design and directed the interpretation of results and manuscript preparation; and all authors: read and approved the final manuscript. None of the authors declared a conflict of interest.

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