JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Metabolism of Anthocyanins by Human Gut Microflora and Their Influence on Gut Bacterial Growth

Maria Hidalgo,[†] M. Jose Oruna-Concha,[‡] Sofia Kolida,[‡] Gemma E. Walton,[‡] Stamatina Kallithraka,[§] Jeremy P. E. Spencer,[‡] Glenn R. Gibson,[‡] and Sonia de Pascual-Teresa*[,†]

[†]Department of Metabolism and Nutrition, Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN), Spanish National Research Council (CSIC), José Antonio Novais 10, Ciudad Universitaria, E-28040 Madrid, Spain

[‡]Department of Food and Nutritional Sciences, School of Chemistry, Food and Pharmacy, University of Reading, Reading RG6 6AP, U.K.

[§]Wine Institute of Athens, S. Venizelou 1, 14123, Likovrisi, Athens, Greece

ABSTRACT: Consumption of anthocyanins has been related with beneficial health effects. However, bioavailability studies have shown low concentration of anthocyanins in plasma and urine. In this study, we have investigated the bacterial-dependent metabolism of malvidin-3-glucoside, gallic acid and a mixture of anthocyanins using a pH-controlled, stirred, batch-culture fermentation system reflective of the distal human large intestine conditions. Most anthocyanins have disappeared after 5 h incubation while gallic acid remained constant through the first 5 h and was almost completely degraded following 24 h of fermentation. Incubation of malvidin-3-glucoside with fecal bacteria mainly resulted in the formation of syringic acid, while the mixture of anthocyanins resulted in formation of gallic, syringic and *p*-coumaric acids. All the anthocyanins tested enhanced significantly the growth of *Bifidobacterium* spp. and *Lactobacillus*−*Enterococcus* spp. These results suggest that anthocyanins and their metabolites may exert a positive modulation of the intestinal bacterial population.

KEYWORDS: *anthocyanins, microbial metabolism, phenolic acids, gut microbiota, prebiotics*

INTRODUCTION

Anthocyanins are a subgroup of flavonoids that contribute to the blue, purple and red color in many fruits and vegetables. Naturally they are polyhydroxy or polymethoxy derivates of 2-phenylbenzopyrylium often with a sugar moiety attached to the aglycon (anthocyanidins). Most frequent in nature are the glycosides of cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin. The daily intake of anthocyanins in humans has been estimated to be between 3 and 215 mg/day;[1,2] more recent studies suggest an average consumption on the order of 57−69 mg/day.[3,4] Gallic acid, on the other hand, is a polyphenol that is ubiquitous in fruits and vegetables. It is a trihydroxybenzoic acid which exists in nature in its free form but can also be found forming hydrolyzable tannins. More recentrly it has been described as being a metabolite or degradation product deriving from different flavonoids. Gallic acid daily intake in humans has been estimated to be 12 mg/day.[3]

The bioavailability of the intact anthocyanin is a fundamental factor for their physiological functions. Anthocyanins are rapidly absorbed through the stomach and small intestine by different mechanisms that may involve specific enzymes such as bilitranslocases.[5,6] However most studies have shown very low bioavailability of anthocyanins including the original anthocyanins and their conjugated metabolites, glucuronidated and sulfated.[7]

Since only a small part of dietary anthocyanins are absorbed, large amounts of the ingested compounds are likely to enter the colon. In vitro studies have shown deglycosylation and degradation of monoglucosides and diglucosides of anthocyanins due to the activity of colonic microbiota.[8,9] Keppler et al., 2005,[9] reported that cyanidin-3-rutinoside was first transformed by gut microbiota into the corresponding glucoside and then converted to phenolic acids which can be further metabolized by the gut microbiota. The anthocyanin nucleus is broken down and protocatechuic acid is detected as a product of the human colonic bacteria. Also metabolism of the methoxyl derivatives was accompanied by O-demethylation. More recently, it has been shown that malvidin-3-glucoside and delphinidin-3-glucoside are metabolized by intestinal microbiota due to β-glucosidase activity.[10,11]

The large intestine is by far the most colonized region of the digestive tract, with a total population of 10^{11}−10^{12} CFU/mL of contents with more than 500 bacterial species, of which over 99% are anaerobic.[12] Through the process of fermentation, gut bacteria are able to produce a wide range of compounds that have both positive and negative effects on gut physiology as well as other systemic influences. The balance among human gut microbiota has been linked to both beneficial and harmful effects in the host. Putatively beneficial bacteria such as *Bifidobaterium* spp. and *Lactobacillus* spp. are genera which include bacteria that have been observed to contribute to human health at different levels. They have been shown to enhance the gut barrier function, stimulate the host immune system, prevent diarrhea or allergies, participate in the

Received: January 18, 2012
Revised: March 14, 2012
Accepted: March 23, 2012
Published: March 23, 2012

dx.doi.org/10.1021/jf3002153 | J. Agric. Food Chem. 2012, 60, 3882−3890

Anthocyanidins	R_1	R_2
Cyanidin-3-glucoside	OH	H
Peonidin-3-glucoside	OCH_3	H
Delphinidin-3-glucoside	OH	OH
Petunidin-3-glucoside	OCH_3	OH
Malvidin-3-glucoside	OCH_3	OCH_3

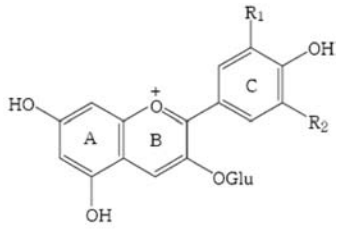

Figure 1. Structure of the main anthocyanidins.

activation of provitamins, and modulate the lipid metabolism.[13−16] However, there are other bacterial species associated with negative implications, such as *Clostridium difficile*, which has been associated with inflammatory bowel disease.[17]

In this study, we have investigated the potential of anthocyanins and gallic acid to influence the growth of specific bacterial groups in a pH-controlled, stirred, batch-culture fermentation system that is reflective of the environmental conditions located in the distal region of the human large intestine. We have also investigated the differential metabolism of all the mentioned compounds in this competitive bacterial environment and show if they are capable of inducing positive changes in the balance of bacterial groups.

MATERIALS AND METHODS

Chemicals. Plant material (grape aqueous extract) obtained as a byproduct of a wine-making industry was kindly provided by Bodegas Osborne and Bodega Capilla del Fraile (Toledo, Spain).

Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich Co. Ltd. (Poole, Dorset, U.K., or Madrid, Spain) or Fisher Scientific (Loughborough, Leics, U.K., or Madrid, Spain).

Bacteriological growth medium supplements were obtained from Oxoid Ltd. (Basingstoke, Hants, U.K.). Enocianin (E-163), a *Vitis vinifera* grape peel anthocyanidin-rich extract, was kindly supplied by Sensient Food Colors Italy (Reggio Emilia, Italy). Enocianin is a commercial food coloring that contains malvidin-3-glucoside (200 mg/L and 20 mg/L respectively), delphinidin-3-glucoside (60 mg/L and 6 mg/L respectively), petunidin-3-glucoside (85 mg/L and 8.5 mg/L respectively), peonidin-3-glucoside (traces), and cyaniding-3-glucoside (traces) (see Figure 1 for structures).

Raftilose P95 FOS was purchased from Orafti (Tienen, Belgium). Anthocyanins used to obtain the calibration curves were purchased by Extrasynthese (Lyon, France).

All the nucleotide probes used for fluorescent in situ hybridization (FISH) were commercially synthesized and labeled with the fluorescent dye Cy3 at the 5′ end (Sigma Aldrich Co. Ltd., Spain).

Sterilization of media and instruments was done by autoclaving at 121 °C for 15 min.

Extraction, Purification, and Isolation of Malvidin-3-Glucoside. In order to isolate the anthocyanin malvidin-3-glucoside, byproduct from the wine industry was used. The aqueous extract was filtered and washed with *n*-hexane, the remaining solvent was removed under vacuum, and the residue was lyophilized. Malvidin-3-glucoside was isolated using a semipreparative RP-HPLC (Waters, Milford, USA) equipped with a double pump system Delta 600 and connected to a 996 photodiode array detector, a 717 plus autosampler injector, and a fraction collector II (Waters). The lyophilized extract (10 g) was diluted with 100 mL of water, filtered through a 0.45 μm filter, and passed through a column Nova Pack HR C18, 4 μm, 300 × 7.8 mm (Waters). Solvents used were aqueous 4.5% formic acid (A) and HPLC-grade acetonitrile (B) at a flow rate of 4 mL/min. Starting with 10% B the gradient was the following: from 10% B to 15% B in 2 min, from 15% to 20% B in 4 min, from 20% to 25% B in 9 min, and isocratically 25% B for 2 min. Detection was carried out at 520 nm. After addition of water, chromatographic solvents were removed under vacuum and the compound was lyophilized and stored at −20 °C.

The purity and identity of malvidin-3-glucoside were checked by HPLC using a diode array detector (HPLC-DAD).

Fecal Sample Preparation. Fecal samples were collected from three separate individuals. All volunteers were in good health and had not ingested antibiotics for at least 6 months before the study. Samples were collected, on site, on the day of the experiment and were used immediately. The samples were diluted 1:10 (w/v) with anaerobic phosphate buffered saline (0.1 M; pH 7.4) and homogenized in a stomacher for 2 min (460 paddle beats/min). Resulting fecal slurries from each individual were used to inoculate the batch-culture vessels.

Batch-Culture Fermentation. Batch-culture fermentation vessels (100 mL volume: one vessel per treatment group) were sterilized and filled with 54 mL of basal nutrient medium (peptone water (2 g/L), yeast extract (2 g/L), NaCl (0.1 g/L), K_2HPO_4 (0.04 g/L), KH_2PO_4 (0.04 g/L), $NaHCO_3$ (2 g/L), $MgSO_4{\cdot}7H_2O$ (0.01 g/L), $CaCl_2{\cdot}6H_2O$ (0.01 g/L), Tween 80 (2 mL/L), hemein (50 mg/L), vitamin K1 (10 mL/L), L-cysteine (0.5 g/L), bile salts (0.5 g/L), resazurin (1 mg/L), and distilled water). The pH of the basal medium was adjusted to 7 and autoclaved before dispensing into vessels. Medium was then gassed overnight with O_2-free N_2 (15 mL/min). Before addition of the fecal slurries, the temperature of the basal nutrient medium was set to 37 °C by using a circulating water bath and the pH was maintained at 6.8 using a pH controller (Electrolab, U.K.). The vessels were inoculated with 6 mL of fecal slurry (1:10, w/v), and in order to mimic conditions located in the distal region of the human large intestine the experiment was run under anaerobic conditions, 37 °C and pH 6.8−7.0 for a period of 24 h. During this period, samples (4 or 3 mL) were collected at four time points (0, 5, 10, and 24 h) for FISH, and at seven time points (0, 1, 2, 4, 5, 10, and 24 h) for anthocyanins and metabolite analysis by HPLC. Before FISH analysis, duplicate samples were fixed for a minimum of 4 h at 4 °C with 4% (w/v) filtered paraformaldehyde. For anthocyanin analysis, aliquots (500 μL) were dispensed into eppendorf tubes containing 500 μL of HCL (0.1% v/v) in methanol and centrifuged at 13000*g* for 10 min. The supernatant fraction was removed and stored at −80 °C. The remaining sample was dispensed (1 mL in duplicate) into eppendorf tubes containing 30 μL of phosphoric acid in water 1:1 (v/v) and centrifuged at 13000*g* for 10 min. The supernatant fraction was removed and stored at −80 °C until analysis.

Inoculation of Substrate in the Batch Culture. Gallic acid (150 mg/L and 1000 mg/L), malvidin-3-glucoside (20 mg/L and 200 mg/L), and enocianin (4850 mg/L and 48500 mg/L) were inoculated in stirring batch-culture vessels (one per treatment) containing fecal slurry (1%). These amounts were estimated to reflect lower and upper levels of malvidin-3-glucoside and gallic acid intake. The higher and lower amounts of enocianin contain the higher (200 mg/L) and lower (20 mg/L) amounts of malvidin-3-glucoside used in our experimental conditions.

The prebiotic FOS (1%, w/v) (Raftilose P95) was included as control in the experiment. Fermentations with no added substrate were also included. All the experiments were carried out in the dark to avoid anthocyanin degradation.

Bacterial Enumeration. In order to assess the changes in bacterial populations fluorescent in situ hybridization (FISH) was used with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA. The probes were commercially synthesized and labeled with the fluorescent dye Cy3. The bacterial groups studied for enumeration were EUB338/II/III, for the total number of bacteria;[18]

Bif 164, specific for *Bifidobacterium* spp.;[19] Lab 158, for *Lactobacillus-Enterococcus* spp.;[20] Erec 482, for the *Clostridium coccoides–Eubacterium rectale* group;[21] Chis 150, for the *C. histolyticum* group;[21] Bac 303, for *Bacteroides* spp.;[22] and Ato 291, for *Atopobium–Coriobacterium* group.[23] Probe sequences and hybridization conditions are listed in Table 1. Batch culture supernatant samples (375 μL) fixed in 1.125 μL of 4% (w/v) filtered paraformaldehyde overnight at 4 °C were then centrifuged at 13000*g* for 5 min, washed twice with PBS (0.1 M; pH 7.0), resuspended in 300 μL of PBS–99% ethanol mixture (1:1, v/v), and stored at −20 °C for at least 1 h. Hybridization of cell suspensions was performed with the use of the modified well method described by Daims et al., 2005.[24] Briefly, the cell suspension was diluted appropriately for each probe and 20 μL was pipetted onto each well of a Teflon- and poly-L-lysine-coated 6-well slide (Tekdon Inc., Myakka City, FL). Slides were dried in a desktop plate incubator for 15 min, dehydrated in ethanol (50%, 80%, and 96% (v/v) ethanol, 3 min each), and dried again. To increase cell permeability for the Lab158, Bif164, and Ato291 probes, samples were treated with 50 μL of lysozyme solution (1 mg/mL in 100 mM Trizma HCl pH 8.0) at room temperature for 15 min before being washed in water and were finally dehydrated in the ethanol series (50%, 80%, and 96% (v/v) ethanol, 3 min each).

Table 1. Fish Oligonucleotide Probes Used in This Study

probe name	target bacterial group/species	sequence from 5′ to 3′ end	T_H (°C)	refs
Ato 291	*Atopobium* spp.	GGT CGGT CT CT CAACCC	50	23
Bac 303	*Bacteroides* spp.	CCAAT GT GGGGGACCT T	46	22
Bif 164	*Bifidobacterium* spp.	CAT CCGGCAT TACCACCC	50	19
Chis 150	*C. histolyticum* group	TT AT GCGGT AT TAATCT YCCT TT	50	21
Erec 482	*Eubacterium rectale–Clostridium coccoides* group	GCT TCTT AGTCARGTACCG	50	21
Lab 158	*Lactobacillus–Enterococcus* spp.	GGT AT TAGCAYCT GTT T CCA	50	20
EUB338	total bacteria	GCT GCCT CCCGT AGGAGT	46	18
EUB338II	total bacteria	GCAGCCACCCGT AGGTGT	46	18
EUB338III	total bacteria	GCT GCCACCCGT AGGTGT	46	18

The hybridization mixture (50 ng/mL probe in hybridization buffer) was added onto the surface of each well, and slides were placed onto the slide tray, which was sealed and left for 4 h in the hybridization oven set at the appropriate temperature for each probe (Table 1). Once the hybridization was complete, slides were removed from the slide tray and transferred into 50 mL of wash buffer (warmed at the appropriate temperature for each probe) containing 20 μL of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 50 ng/mL; Sigma) for a further 15 min. Slides were then dipped into ice-cold distilled water for 2–3 s and dried with a stream of compressed air. Antifade solution (polyvinyl alcohol mounting medium with DABCO antifading; Sigma) was then added to each well, and a coverslip was applied. Slides were stored in the dark at 4 °C (for a maximum of 3 days) until cells were counted under an epifluorescence microscope (Brunel Ltd., Wiltshire, U.K.). The DAPI-stained cells were examined under ultraviolet light, and hybridized cells were viewed with the use of a DM510 filter. For each slide, at least 15 random different fields of view were counted. The following equation was used to calculate the numbers of bacteria: log10 ($C = N \times 0.8 \times 8702.47 \times 50q$), where C is cell/mL of sample, N is the average count derived from 15 fields of view, 8702.47 is the magnification constant, 50 is used to work the value back to 1 mL at 20 μL used on the slide, q is the dilution factor, and 0.8 is the dilution factor for the FISH preparation step. Microbial counts were expressed as $\log_{10}$ bacterial cells per mL of feces.

HPLC Analysis. Quyantitative analysis of the disappearance of anthocyanin glycosides and gallic acid and the formation of anthocyanin metabolites was carried out using an Agilent 1200 series liquid chromatograph with a quaternary pump and a photodiode array detector. Samples were centrifuged (10000 rpm/5 min/4 °C), and the supernatants were filtered through a 0.45 μm filter and autoinjected into the HPLC system. HPLC-DAS was performed using an Ultrabase C18 column (5 μm; 4.6 mm × 150 mm) which was set thermostatically at 25 °C. Solvents used to analyzed disappearance of anthocyanins were aqueous 4.5% formic acid (A) and HPLC-grade acetonitrile (B) at a flow rate of 0.5 mL/min. Starting with 10% B, the gradient was the following: from 10% B to 20% B in 20 min, from 20% B to 25% B in 10 min, from 25% B to 35% B in 10 min, and isocratically 35% B in 10 min. Detection wavelengths were 280, 330, 370, and 520 nm. Anthocyanins were detected at 520 nm, and their peak areas were referred to calibration curves obtained with malvidin-3-glucoside, delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, and peonidin-3-glucoside. Limits of detection and quantification were calculated and were in every case below 0.1 μg/mL.

Solvents used to quantitatively analyze other phenolic compounds were acetic acid 2.5% (A), HPLC-grade acetonitrile (B), ultrapure water (C), and acetic acid 2.5%/ HPLC-grade acetonitrile (90:10) (D) at a flow rate of 0.5 mL/min. Starting with 100% A, the gradient was the following: from 100% A to 100% D in 3 min, from 100% D to 1% B in 4 min, isocratically 1% B in 3 min, from 1% B to 15% B in 20 min, from 15% B to 60% B in 5 min, and isocratically 60% B in 5 min. Detection wavelengths were 280, 330, and 370 nm. Areas of gallic acid and other identified phenolic compounds were compared with calibration curves prepared with the corresponding standards, wherever possible. Limits of detection and quantification were calculated and were in every case below 0.5 μg/mL.

HPLC–MS Analysis. In order to confirm the identity of the recorded anthocyanins and phenolic compounds and to detect other potential compounds that could not be detected with HPLC-DAS, additional analysis was performed by using HPLC with mass spectrometry detection. Mass spectrometry was performed using an Agilent 1100 series liquid chromatograph equipped with an API source and employing an ESI (electrospray ionization) interface. The HPLC system was connected to a photodiode array detector and a simple quadruple G1946D Q-LC/MS. Sheath as well as auxiliary gas was a mixture of helium and nitrogen. The capillary voltage was 3 V and the capillary temperature 180 °C. Solvents used were ultrapure water (A), HPLC-grade acetonitrile (B), formic acid 1% (C), and formic acid 1%/HPLC-grade acetonitrile (90:10) (D) at a flow rate of 0.5 mL/min. Starting with 100% C, the gradient was the following: from 100% C to 100% D in 3 min, from 100% D to 1% B in 4 min, isocratically 1% B in 3 min, from 1% B to 12% B in 20 min, from 12% B to 50% B in 5 min, isocratically 50% B in 5 min, and from 50% B to 100% C in 2 min. The MS detector was programmed to perform a series of consecutive scans: full scan from m/z 150 to 1500 and in addition MS^2, MS^3, and MS^4 spectra from the most abundant ion were recorded. Spectra were recorded in the positive ion mode. Standards of anthocyanins and other phenolic compounds were also injected to help in the identifications.

Statistical Analysis. Malvidin-3-glucoside, enocianin, and gallic acid were tested in batch cultures inoculated with fecal samples collected from three individual donors in three separate experiments. The $\log_{10}$ numbers of specific bacteria were expressed as mean values and standard deviations. Statistical tests were performed using the SPSS computer program, version 19.0 (SPSS Statistical Software, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA): post hoc multiple comparisons was carried out. Differences between control and tested samples were assessed on the basis of confidence intervals using the Tukey test. The level of significance was $p \leq 0.05$*).

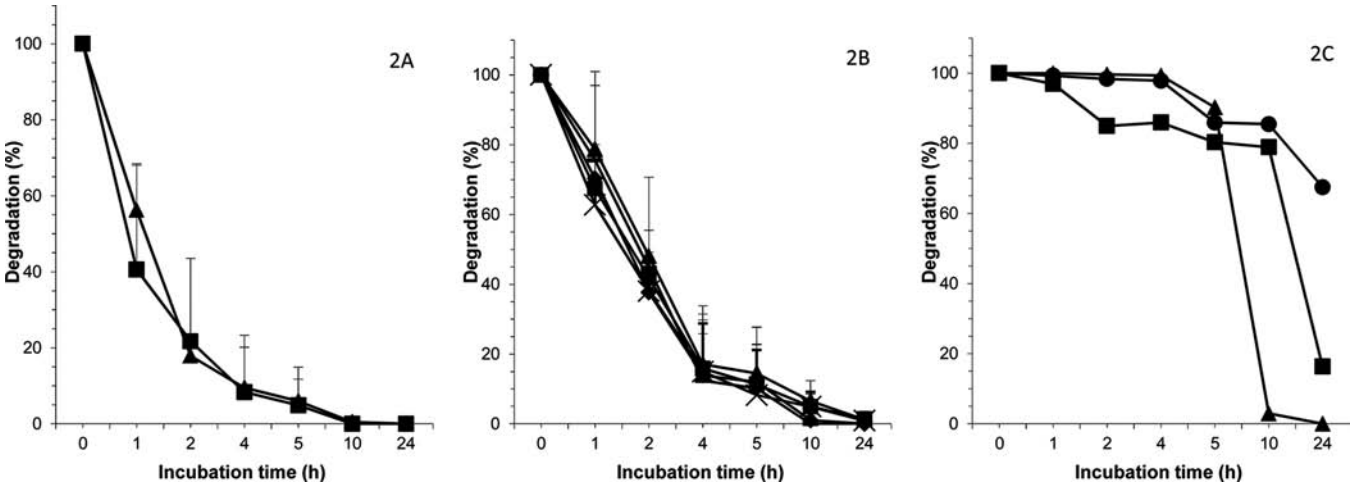

Figure 2. Percentage of degradation (y axes) of malvidin-3-glucoside (200 mg/L) (▲) and malvidin-3-glucoside (20 mg/L) (■) (A); enocianin 4850 mg/L: malvidin-3-glucoside (■), delphinidin-3-glucoside (▲), cyanidin-3-glucoside (rectangle), petunidin-3-glucoside (×), and peonidin-3-glucoside (◆) (B), and gallic acid 1000 mg/L tested with donor 1 (▲), donor 2 (●), and donor 3 (■) (C) in the presence of gut microflora. Data were derived from three independent experiments, and values are expressed as means ± SD, except in panel C, where the three experiments are showed individually.

RESULTS AND DISCUSSION

Metabolism of Anthocyanins and Gallic Acid by Human Fecal Microbiota. In order to evaluate the metabolism of gallic acid, malvidin-3-glucoside, or the mixture of anthocyanins containing malvidin-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside, and cyanidin-3-glucoside by human fecal microbiota, incubation media collected at 0, 1, 2, 4, 5, 10, and 24 h were analyzed using HPLC-DAD to identify and quantify the metabolites. Additionally, HPLC−MS was used to confirm phenolic products.

Anthocyanins and Gallic Acid Disappearance from the Fermentation Media. After 1 h of incubation, malvidin-3-glucoside incubated alone was greatly metabolized in the higher (44%) and lower (59%) concentrations, and almost completely metabolized after 4 h of incubation (Figure 2A). All anthocyanins contained in the mixture at lower concentration underwent a great metabolism (85%) within the first 4 h and were totally degraded following 24 h of fermentation (Figure 2B), with the exception of batch culture 3, where a small amount (1.23%) still remained at 24 h. In the mixture at high concentration, anthocyanins were greatly metabolized within 4 h (46%) and were almost completely degraded at 24 h, except in batch culture 3, where a residual amount (31%) of malvidin-3-glucoside was still present (data not shown). The fastest rate of degradation of malvidin-3-glucoside alone versus malvidin-3-glucoside mixed with other anthocyanins could be due to the complicated bacterial access to the matrix sample, and this could potentially reflect a more realistic approach of what may be happening in vivo in the human colon.

On the other hand, gallic acid concentration in the medium remained constant within the first 5 h of microbial fermentation with the highest rate of metabolism taking place between 5 and 24 h, after which it was almost completely degraded. Gallic acid, however, underwent a slower metabolism rate in batch 2, and approximately 50% of the initial concentration still remained at 24 h (Figure 2C).

Incubation of malvidin-3-glucoside without fecal microbial fermentation confirms chemical stability at pH 6.5 and 37 °C, probably due to the presence of glucose and the methoxylation pattern that lead to a high stability.[10] However, chemical degradation of delphinidin-3-glucoside demonstrated 60% of disappearance within the first 5 h of fermentation and was almost fully degraded by 24 h. The presence of 3 hydroxyl groups in the B-ring structure makes it susceptible to chemical degradation; moreover stability is affected by pH, temperature, light, and oxygen.

Appearance of Newly Formed Compounds after Anthocyanin and Gallic Acid Metabolism. Chemical breakdown of delphinidin leads to the formation of gallic acid and traces of homogentisic, syringic, and *p*-coumaric acids.[10] Moreover, our results indicate that the degradation of malvidin-3-glucoside is entirely due to specific bacterial-dependent metabolism; in contrast delphinidin-3-glucoside reduction is unspecific bacterial-independent degradation.

No aglycons were found for any of the anthocyanin glycosides used under any of the collection times assayed. Anthocyanin glycosides must have been hydrolyzed by enzymes of fecal microbiota by cleavage of the 3-glycosidic linkage; the released aglycons formed transitorily could have been degraded into the corresponding phenolic acids emanating from B ring since aglycons are very unstable under physiological conditions in the intestine at neutral pH. However, Keppler and Humpf, 2005,[9] detected small amounts <10 μM of aglycons after 2 h of incubation with pig gut microbiota. Our data appear to suggest that the formation of imine may have occurred as a result of the high reactivity of aldehydes with free NH_2 groups of amino acids or proteins.

Anthocyanin recovery from fecal slurries was calculated to be 85%, 60%, and 90% respectively for malvidin-3-glucoside, the mixture of anthocyanins, and gallic acid respectively. It is possible that the losses are due to the attachment to the fecal slurry of the different anthocyanins. This last could clarify why low amounts of primary phenolic products appear in 0 h samples with malvidin-3-glucoside alone or mixed with other anthocyanins. The main primary phenolic degradation product detected in incubation with malvidin-3-glucoside was syringic acid (t_R = 19.2). The highest amounts of syringic acid were observed after 4 h of incubation (Figure 3), and after that the amount decreased gradually in parallel to the rate of degradation of malvidin-3-glucoside (Figure 2A). The metab-

dx.doi.org/10.1021/jf3002153 | J. Agric. Food Chem. 2012, 60, 3882−3890

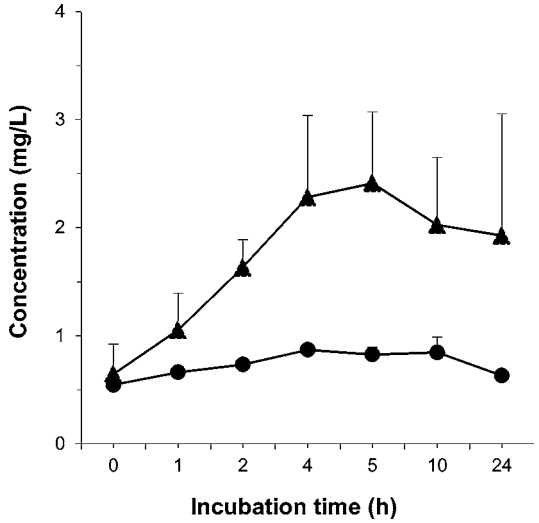

Figure 3. Formation of syringic acid from malvidin-3-glucoside (200 mg/L) (▲) and malvidin-3-glucoside (20 mg/L) (●) in the presence of gut microflora. Data were derived from three independent experiments, and values are expressed as means ± SD.

olism of malvidin-3-glucoside also transitorily provided high amounts of gallic acid (t_R = 7.6) and pyrogallol (t_R = 7.1). Syringic acid might have suffered enzymatic demethylation of C3 and C5 of the B-ring, which degenerated into gallic acid, even reaching a higher amount than syringic acid. The fact that pyrogallol was found in the sample indicates further metabolism such as decarboxylation of the first metabolite, gallic acid.

As Table 2 indicates, more peaks were detected in the HPLC profile following incubation with malvidin-3-glucoside (Figure 4). However, taking into account the features and the corresponding spectrum, it is possible to assume that these peaks could belong to benzoic acids following further metabolism, since hydroxylated aromatic compounds are formed from the A-ring. Some authors have also identified hydroxylated and nonhydroxylated benzoic and phenylacetic derivatives from other flavonoids.[25−28]

Table 2. Chromatographic and Spectral Characteristics of Anthocyanin Metabolites Produced by Gut Microbiota

peak	name	t_R (min)	λ_{max} (nm)	molecular ion M^+
1	unknown	4.2	298	125
2	unknown	4.7	278	123
3	unknown	6.5	274	139
4	pyrogallol	7.1	264	127
5	gallic acid	7.7	272	171
6	unknown	10.5	270	123
7	unknown	18.5	322	371
8	syringic acid	19.3	272	199
9	*p*-coumaric acid	25.6	308	165

With respect to anthocyanin mixtures, as expected, mainly formation of gallic, syringic, and *p*-coumaric acids took place. As shown in Figure 5, a great amount of gallic acid was detected at low sample concentrations, reaching the maximum concentration at 4−5 h, a time where anthocyanins were extensively degraded. Both chemical and microbiologic degradation of delphinidin-3-glucoside seem to be responsible for the formation of their corresponding phenolic acids. Besides

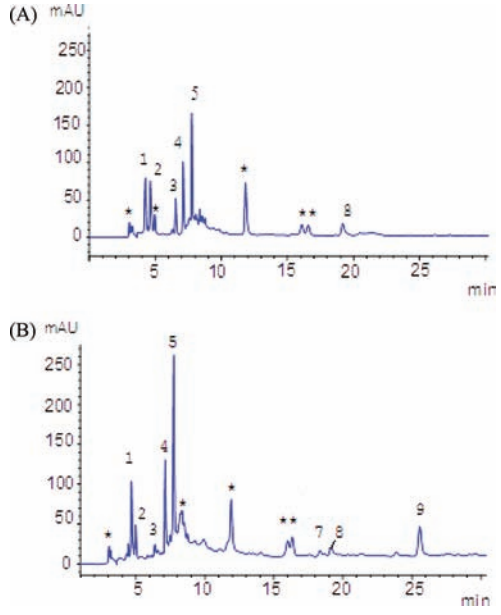

Figure 4. HPLC chromatograms recorded at 280 nm corresponding to malvidin-3-glucoside (200 mg/L) at 4 h (A) and enocianin (4850 mg/L) at 5 h (B). Peak identities are detailed in Table 2. The asterisks represent the peaks that also appear in the control without substrate.

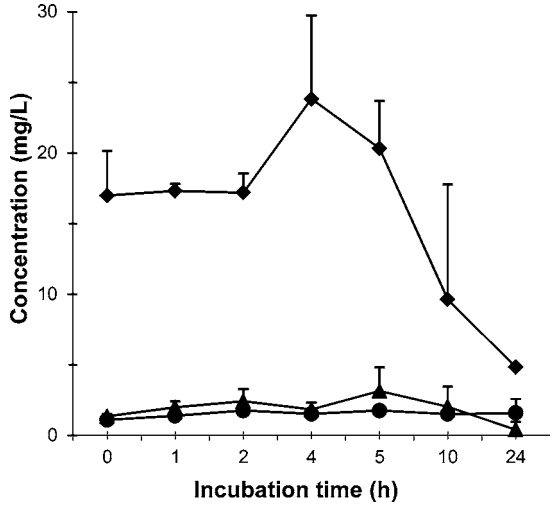

Figure 5. Formation of gallic (◆), syringic (●), and *p*-coumaric acid (▲) from enocianin (4850 mg/L) in the presence of gut microflora. Data were derived from three independent experiments and values are expressed as means ± SD.

simultaneous deglycosylations of malvidin-3-glucoside, enzymatic reactions must have taken place to transform gallic acid whereas the metabolism of anthocyanins might have contributed toward the formation of syringic and *p*-coumaric acid.

The addition of excess substrate in the experiments with high concentration of anthocyanins appeared to preserve them from bacterial metabolism, in fact we only detected syringic and *p*-coumaric acid with the second donor and gallic and *p*-coumaric acids with the third donor.

According to other studies,[9,29] incubation of gut microbiota with malvidin-3-glucoside led to the formation of syringic acid as the main degradation product. Regarding the degradation of delphinidin-3-glucoside, gallic acid was also formed, however, in a previous study where delphinidin-3-glucoside was incubated

with pig gut microbiota, gallic acid was not found, indicating that this phenolic acid could have been metabolized.[30] Fleschhut et al.[29] identified vanillic and protocatechuic acids as the major degradation products of peonidin-3-glucoside and cyanidin-3-glucoside, respectively, after incubation with human feces. In our study, we might not have detected these degradation products due to the initial low amount of both anthocyanins in the sample

On the other hand, gallic acid is rather stable against further metabolism by human gut microbiota. We observed the formation of pyrogallol following 10 h fecal fementation with the first donor in both concentrations whereas with the second and third donors it was detected following 24 h of fermentation. Furthermore, we noticed that the appearance of pyrogallol at 10 h took place with donor 1 where gallic acid was degraded more rapidly.

The rate of degradation and transformation of the tested compounds differed in batch cultures inoculated with samples of different fecal donors. Therefore, we could conclude that the individual composition of intestinal microbiota contributes to the bioavailability and bioefficacy of phenolic compounds in the systemic circulation. Consequently, the colon may be considered as an active site of metabolism in which bacteria contribute to the health effects of phenolic compounds.

In general, glycosylated anthocyanins were rapidly degraded by the fecal microbiota, and more rapidly following incubation of individual ones such as malvidin-3-glucoside. However, the disappearance of anthocyanins during the incubation did not correspond with the amount of phenolic acids formed. Hence, other reactions might contribute to anthocyanin disappearance. The flavylium form of anthocyanins is reversible at neutral pH and could be transformed into the quinoidal anhydrobase, which may react with different groups in several macromolecules.

Our results suggest that anthocyanins, such as malvidin-3-glucoside, which are poorly absorbed after ingestion, enter the colon and are fermented by the intestinal microbiota to produce anthocyanin metabolites which might be responsible for observed health effects in vivo.[31] In terms of biological activity, the microbial metabolism could improve the health-giving properties. Previous studies have reported that gallic acid exerts antioxidant activity against oxidative stress, and in vitro studies have confirmed that gallic acid exhibits antiadhesive, antiproliferative, and angiotensin-converting enzyme inhibitory activities.[32] With regard to syringic acid, studies carried out by Stanikunaite et al., 2009,[33] showed that it displays a strong antioxidant activity as well as an inhibitory COX-2 activity in mouse macrophages (RAW 264.7). In addition, phenolic acids such as *p*-coumaric are observed to exert anti-inflammatory activity in human colonic fibroblasts.[34] However, some health effects of microbial metabolites do not require their absorption through the gut barrier and therefore they may have a direct impact on the gut mucosa and protect it against oxidative stress or the action of carcinogens.[35]

Anthocyanins and Gallic Acid-Induced Changes in Specific Bacterial Groups. In order to evaluate the changes in bacterial populations in response to malvidin-3-glucoside, enocianin, and gallic acid, we used FISH analysis. This method has been previously used in in vitro and in vivo studies to assess potential prebiotic effects of different substrates.[37,51,52] FISH analyses were performed at 0 (control), 5, 10, and 24 h of incubation. Malvidin-3-glucoside enhanced the growth of total bacteria achieving a statistically significant increase at 24 h of incubation when tested at high concentration (Figure 6A), compared with the total number measured in the control vessel

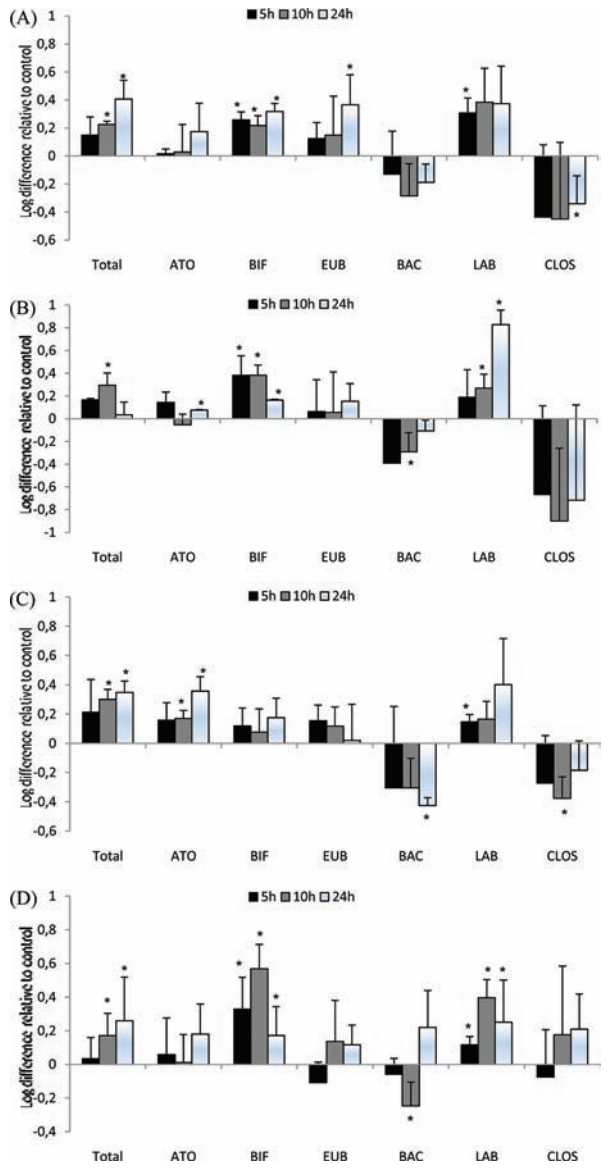

Figure 6. Influence of malvidin-3-glucoside (200 mg/L) (A), enocianin (4850 mg/L) (B), gallic acid (1000 mg/L) (C), and FOS (1%, w/v) (D) on the gut microflora in a pH-controlled fecal batch culture. Samples were collected at 0, 5, 10, and 24 h and analyzed by fluorescence in situ hybridization. ATO, *Atopobium* spp.; BIF, *Bifidobacterium* spp.; EUB, *C. cocoides*−*Eubacterium rectale* group; BAC, *Bacteroides* spp.; LAB, *Lactobacillus* spp.; CLOS, *Clostridium histolyticum* group. Changes in bacterial growth calculated by comparing the number of a specific bacterial group in the treatment with the number found in a control group, at the same time point. Values are mean (n = 3), with standard deviations represented by vertical bars. Mean value was significantly different from that of the control: $^{*}p < 0.05$.

(log 8.99 ± 0.19). Furthermore, malvidin-3-glucoside caused a significant increase in the growth of bacteria associated with beneficial effects such as *Bididobacterium* spp. and *Lactobacillus* spp. in relation to the control vessel at the same time points. With respect to *Bifidobacterium* spp., the highest concentration of malvidin-3-glucoside showed statistically significant increases at 5, 10, and 24 h (Figure 6A) compared with the total number

dx.doi.org/10.1021/jf3002153 | J. Agric. Food Chem. 2012, 60, 3882−3890

measured in controls at 5, 10, and 24 h (log 7.87 ± 0.13, 7.87 ± 0.10 and 7.76 ± 0.18 respectively). A tendency to an increase was shown in the growth of the microbial group of *C. coccoides*–*Eubacterium rectale* at 24 h—a group associated with butyrate production. A decrease, although not significative, in the potentially detrimental *C. histolyticum* group was observed following 24 h fermentation. No significant effect in the growth of *Bacteroides* spp. measured in vessels supplemented with malvidin-3-glucoside was shown. Therefore overall a selective shift in the microbial community was observed that could offer potential benefits to the host.

The addition of high concentration level of mixed anthocyanins to the vessels rendered the microbial cells impossible to count, therefore only data from vessels supplemented with low concentration of enocianin is presented.

The vessels supplemented with the mixture of anthocyanins at low concentration caused a marked significant increase in the growth of *Bifidobacterium* spp. following 5, 10, and 24 h fermentation, reaching a higher growth than in the presence of malvidin-3-glucoside. Moreover, the noticeable growth of *Lactobacillus* spp. observed in the presence of blended anthocyanins was statistically significant following 5, 10, and 24 h fermentation (controls for 5, 10, and 24 h were log 7.34 ± 0.15, 7.21 ± 0.22, and 7.09 ± 0.35 respectively). Furthermore, anthocyanin exposure resulted in greater growth stimulation of *Lactobacillus* spp. than the positive control FOS, a well-known commercial prebiotic. The total bacterial number in media treated with anthocyanins increased significantly in relation to the control after 10 h of fermentation (log 9.05 ± 0.05). However, there was no significant change in *C. coccoides*–*Eubacterium rectale* group or *C. histolyticum* group.

In addition, we tested one of the most important metabolites of anthocyanins, gallic acid. In general, gallic acid reduced the growth of a group that includes some potentially harmful bacterial *C. histolyticum* group without negative effect on beneficial bacteria tested (Figure 6C). Both concentrations of gallic acid reduced consistently the growth of *C. histolyticum* group. It was significantly reduced at 10 h in high concentrations and at 10 and 24 h in low concentrations, moreover it significantly inhibited the growth of *Bacteroides* spp. at 10 and 24 h in high concentration tested and at 24 h in the low concentration (control values at 10 and 24 h were log 8.44 ± 0.12 and 8.25 ± 0.06 respectively). Additionally, gallic acid significantly enhanced the growth of the total bacterial number and *Atopobium* spp. following 24 h fermentation (log 7.75 ± 0.10 for the control) (Figure 6C).

The significant changes in bacterial composition in response to the rapid metabolism to malvidin-3-glucoside is especially marked with the higher amount, however when we added the lower amount of malvidin-3-glucoside mixed with other anthocyanins, we found a synergistic effect with respect to the enhancement of growth of the beneficial bacterial group comparing with both concentrations of individual malvidin-3-glucoside supplementation. In contrast, the significant changes in bacterial composition in response to gallic acid are similar in both concentrations. Its later degradation and the differences obtained in the rate of metabolism among different donors could be responsible for this effect.

Current evidence supports the concept that oral administration of probiotic therapies may be beneficial in gastrointestinal tract disorders. Probiotics have been defined as a live microbial ingredient the beneficial effects of which in the gastrointestinal tract, including enhancement of gut barrier function, regulation of immune system, improving atopic diseases, diarrhea or vaginal infections, are well-known.[36] The selectively stimulating growth or activity of one or a limited number of beneficial bacteria in the colon is trigged by prebiotics. Both inulin and FOS have been already demonstrated to be effective prebiotics.[37] Our data show that when anthocyanins are fermented and the metabolites are available in the large bowel, modulation of the composition of the bacterial population is observed, especially in *Lactobacillus* spp. and *Bifidobacterium* spp. The rapid metabolism of anthocyanins occurred over the first 4 to 5 h in all fermentation experiments, and it was not surprising since major groups of intestinal bacteria possess β-glucosidase activity, including *Lactobacillus* spp. and *Bifidobacterium* spp. These bacteria possess the ability to metabolize phenolic compounds during growth supplying energy to cells and enriching the medium for bacterial growth with the release of glucose. Therefore, anthocyanins, particularly the grape derived mixture of anthocyanins, might act as growth-stimulator of *Lactobacillus* spp. and *Bifidobacterium* spp. which are associated with beneficial effects in the large intestine including the antimicrobial effect of pathogenic microorganisms by production of short chain fatty acids, as well as by competition for growth substrate and adhesion sites.[38] Increasing the numbers of lactic acid bacteria in the colon has been found to reduce the formation of procarcinogens in the large intestine[39] and to reduce the pH value. Moreover, the short chain fatty acids generated by these bacteria are linked to an inhibition of preneoplasic proliferation, conversion of cholesterol into bile acids, and modulation of the expression of multiple genes involved in the atherosclerosis process.[17,40,41] In addition, malvidin-3-glucoside showed a tendency to promote the growth of *C. coccoides*–*Eubacterium rectale*, a group of bacteria known to produce large amounts of butyrate, for which anti-inflammatory and antineoplasic properties have been demonstrated in vitro, potentially benefiting inflammatory bowel disease and colorectal cancer sufferers.[42,43] In contrast, the blend of anthocyanins containing malvidin-3-glucoside did not achieve an effect in the growth of this bacterial group. We also found that *Atopobium* spp. increased after supplementation with anthocyanins as well as with gallic acid. The role of *Atopobium* spp. in the human colon is not clear. Although they have been isolated from healthy human feces and increase after ingestion of the commercial prebiotic inulin, little is known about their effects. Some species can ferment glucose to organic acids as well as ferment peptides or amino acids. In a recent study, *Atopobium minutum* has been shown to induce apoptosis in colonic cancer cells and therefore suggest that this bacterium could act in a positive way.[44]

Moreover we have found a marked decrease of *C. histolyticum*, a proteolytic bacterium, associated with tumor promoting properties and inflammatory bowel disease,[13,45] when we supplemented the medium with gallic acid reaching significant inhibition at 10 and 24 h.

In previous studies, gallic acid as well as other benzoic acids and phenolic extracts exerted antimicrobial properties against pathogenic bacteria such as *Escherichia coli, Clostridium perfringens, Staphyloccocus aureus, Helicobacter pylori*, or *Salmonella enterica*[46−49] without significantly affecting the growth of beneficial bacteria. In our study, interestingly gallic acid inhibited the growth of potentially negative bacteria without inhibiting any of the putatively beneficial bacteria tested. It is well-known that gallic acid is a good iron chelator

that forms stable complexes with these metal ions and hence decreases their availability to bacteria. Moreover, phenolic acids decrease the pH environment and form complexes with proteins or metal ions.

According to our results, studies in both humans and animals have revealed an increase in the growth of potentially beneficial bacteria such as *Lactobacillus* spp., *Bifidobacterium* spp., or the butyrate producing *C. coccoides*−*Eubacterium rectale* after administration of blackcurrant extract powder,[50] pomegranate extracts,[51] or flavanol monomers.[52] Moreover, polyphenols may act as antimicrobial compounds. Therefore, polyphenols such as the anthocyanins tested in this study may have potential health benefits via modulation of the gut microbiota.

In conclusion, despite the association between anthocyanin consumption and human health in a great number of epidemiological studies, their bioavailability is a key issue in their physiological function. It has been proved that anthocyanins can reach the colon in large amounts and thus gut microbiota might play a crucial role in their bioactivity.[31] The present work provides new findings about the interaction between anthocyanins and gut microbiota which could act to explain the health effects observed after intake of anthocyanin-rich diets. Bacterial metabolism involves the cleavage of glycosidic linkages, breakdown of anthocyanidin heterocycle, and a further metabolism that can transform anthocyanins to smaller phenolic compounds in the colon. New metabolites like gallic acid, syringic acid, *p*-coumaric acid, and pyrogallol appeared after bacterial metabolism of anthocyanins, some of which have been described as being more bioactive than the native molecule. These observations suggest that anthocyanins may be potential nutrient sources for gut bacteria; moreover they and their metabolites may contribute to the maintenance of gastrointestinal health by exerting antioxidant activity in the large intestine and by modulating the gut microbial composition. Our data indicate that anthocyanins and their metabolites exert a potentially beneficial effect in the intestinal bacterial flora. However, future studies on the metabolism of anthocyanins and the role played in the potential prebiotic effect need to be performed in vivo.

AUTHOR INFORMATION

Corresponding Author

*Tel: +34 915492300. Fax: +34 915493627. E-mail: soniapt@ictan.csic.es.

Funding

This work has been financially supported by the Spanish Ministry of Science and Innovation through Projects AGL2006-05453 and AGL2009-07894 and the Consolider-Ingenio 2010 Programme (FUN-C-FOOD, CSD2007-00063), a CSIC predoctoral fellowship to M.H. (JAEPre094), and a CSIC Mobility Program fellowship to S.d.P.-T. (PA1002608).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Chun, O. K.; Chung, S. J.; Song, W. O. Estimated dietary flavonoid intake and major food sources of U.S. adults. *J. Nutr.* **2007**, *37*, 1244−1252.

(2) Kuhnau, J. The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev. Nutr. Diet.* **1976**, *24*, 117−191.

(3) Pérez-Jiménez, J.; Fezeu, L.; Touvier, M.; Arnault, N.; Manach, C.; Hercberg, S.; Galan, P.; Scalbert, A. Dietary intake of 337 polyphenols in French adults. *Am. J. Clin. Nutr.* **2011**, *93* (6), 1220−1228.

(4) Beking, K.; Vieira, A. An assessment of dietary flavonoid intake in the UK and Ireland. *Int. J. Food Sci. Nutr.* **2011**, *62*, 17−19.

(5) Talavera, S.; Felgines, C.; Texier, O.; Besson, C.; Lamaison, J. L.; Remesy, C. Anthocyanins are efficiently absorbed from the stomach in anesthetized rats. *J. Nutr.* **2003**, *133*, 4178−4182.

(6) Passamonti, S.; Vrhovsek, U.; Vanzo, A.; Mattivi, F. The stomach as a site for anthocyanins absorption from food. *FEBS Lett.* **2003**, *544*, 210−213.

(7) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Rémésy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230−242.

(8) Aura, A. M.; Martin-López, P.; O'Leary, K. A.; Williamson, G.; Oksman-Caldentey, K. M.; Poutanen, K.; Santos-Buelga, C. In vitro metabolism of anthocyanins by human gut microflora. *Eur. J. Nutr.* **2005**, *44*, 133−142.

(9) Keppler, K.; Humpf, H. U. Metabolism of anthocyanis and their phenolic degradation products by the intestinal microflora. *Bioorg. Med. Chem.* **2005**, *13*, 5195−5205.

(10) Ávila, M.; Hidalgo, M.; Sánchez-Moreno, C.; Pelaez, C.; Requena, M. T.; de Pascual-Teresa, S. Bioconversion of anthocyanin glycosides by *Bifidobacteria* and *Lactobacillus*. *Food Res. Int.* **2009**, *42*, 1453−1461.

(11) Selma, M. V.; Espín, J. C.; Tomás-Barberán, F. A. Interaction between phenolics and gut microbiota: role in human health. *J. Agric. Food Chem.* **2009**, *57*, 6485−6501.

(12) Macfarlane, S.; Macfarlane, G. T. In *Gut Flora, Nutrition, Immunity and Health*; Fuller, R., Perdigon, G., Eds.; Blackwell Publishing: Oxford, U.K., 2003; pp 24−51.

(13) Gibson, G. R. Prebiotics as gut microflora management tools. *J. Clin. Gastroenterol.* **2008**, *42*, 75−79.

(14) Hord, N. G. Eukaryotic-microbiota crosstalk: potential mechanisms for health benefits of prebiotics and probiotics. *Annu. Rev. Nutr.* **2008**, *28*, 215−231.

(15) Tannock, G. W. Probiotics and prebiotics: Where are we going? In *Probiotics and prebiotics: Where are we going?* Tannock, G. W., Ed.; Caister Academic Press: Wymondham, 2002; pp 1−20.

(16) Reddy, B. S. Possible mechanisms by which pro- and prebiotics influence colon carcinogenesis and tumor growth. *J. Nutr.* **1999**, *129*, 1478−1482.

(17) Rastall, R. A.; Gibson, G. R.; Gill, H. S.; Guarner, F.; Klaenhammer, T. R.; Pot, B.; Reid, G.; Rowland, I. R.; Sanders, M. E. Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: an overview of enabling science and potential applications. *FEMS Microbiol. Ecol.* **2005**, *52*, 145−152.

(18) Daims, H.; Brühl, A.; Amann, R.; Schleifer, K. H.; Wagner, M. The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: development and evaluation of a more comprehensive probe set. *Syst. Appl. Microbiol.* **1999**, *22*, 434−444.

(19) Langendijk, P. S.; Schut, F.; Jansen, G. J.; Raangs, G. W.; Kamphuis, G. R.; Wilkinson, M. H. F.; Welling, G. W. Quantitative fluorescent in situ hybridisation of *Bifidobacterium* spp. with genus specific 16S rRNA targeted probes and its application in fecal samples. *Appl. Environ. Microbiol.* **1995**, *61*, 3069−3075.

(20) Harmsen, H. J. M.; Elfferich, P.; Schut, F.; Welling, G. W. A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent *in situ* hybridization. *Microb. Ecol. Health Dis.* **1999**, *11*, 3−12.

(21) Franks, A. H.; Harmsen, H. J. M.; Raangs, G. C.; Jansen, G. J.; Schut, F.; Welling, G. W. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group specific 16S rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* **1998**, *64*, 3336−3345.

(22) Manz, W.; Amann, R.; Ludwig, W.; Vancanneyt, M.; Schleifer, K. H. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-

flavobacter-bacteroides in the natural environment. *Microbiology* **1996**, *142*, 1097−1106.
(23) Harmsen, H. J. M.; Wildeboer-Veloo, A. C. M.; Grijpstra, J.; Knol, J.; Degener, J. E.; Welling, G. W. Development of 16S rRNA-based probes for the *Coriobacterium* group and the *Atopobium* cluster and their application for enumeration of *Coriobacteriaceae* in human feces from volunteers of different ages. *Appl. Environ. Microbiol.* **2000**, *66*, 4523−4527.
(24) Daims, H.; Stoecker, K.; Wagner, M. Fluorescence *in situ* hybridization for the detection of prokaryotes. In *Molecular Microbial Ecology*; Osborn, A. M., Smith, C. J., Eds.; Garland Science: Abingdon, U.K., 2005; Chapter 9, pp 213−239.
(25) Winter, J.; Moore, L.; H., Dowell, J. R.; Bokkenheuser, V. D. C-ring cleavage of flavonoids by human intestinal bacteria. *Appl. Environ. Microbiol.* **1989**, *55*, 1203−1208.
(26) Déprez, S.; Brezillon, C.; Rabot, S.; Philippe, C.; Mila, I.; Lapierre, C.; Scalbert, A. Polymeric proanthocyanidins are catabolized by a human colonic microflora into low-molecular-weight phenolic acids. *J. Nutr.* **2000**, *130*, 2733−2738.
(27) Gonthier, M. P.; Cheynier, V.; Donovan, J. L.; Manach, C.; Morand, C.; Mila, I.; Lapierre, C.; Rémésy, C.; Scalbert, A. Microbial aromatic acid metabolites formed in the gut account for a major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. *J. Nutr.* **2003**, *133*, 461−467.
(28) Van't Slot, G.; Humpf, H. U. Degradation and metabolism of catechin, epigallocatechin-3-gallate (EGCG), and related compounds by the intestinal microbiota in the pig cecum model. *J. Agric. Food Chem.* **2009**, *57*, 8041−8048.
(29) Fleschhut, J.; Kratzer, F.; Rechkemmer, G.; Kulling, S. E. Stability and biotransformation of various dietary anthocyanins in vitro. *Eur. J. Nutr.* **2006**, *45*, 7−18.
(30) Forester, S. C.; Waterhouse, A. L. Identification of cabernet sauvignon anthocyanin gut microflora metabolites. *J. Agric. Food Chem.* **2008**, *56*, 9299−9304.
(31) Kahle, K.; Kraus, M.; Scheppach, W.; Ackermann, M.; Ridder, F.; Richling, E. Studies on apple and blueberry fruit constituents: do the polyphenols reach the colon after ingestion? *Mol. Nutr. Food Res.* **2006**, *50*, 18−23.
(32) Hidalgo, M.; Martín-Santamaría, S.; de Pascual-Teresa, B.; Recio, I.; Sánchez-Moreno, C.; de Pascual-Teresa, S. Potential anti-inflammatory, anti-adhesive, anti/estrogenic and angiotensin-converting enzyme inhibitory activities of anthocyanins and their gut metabolites. *Genes Nutr.* **2011**, Ms. No. GENU-D-11-00075R1.
(33) Stanikunaite, R.; Khan, S. I.; Trappe, J. M.; Ross, S. A. Cyclooxygenase-2 inhibitory and antioxidant compounds from the truffle Elaphomyces granulatus. *Phytother Res.* **2009**, *23*, 575−578.
(34) Russell, W. R.; Drew, J. E.; Scobbie, L.; Duthie, G. G. Inhibition of cytokine-induced prostanoid biogenesis by phytochemicals in human colonic fibroblasts. *Biochim. Biophys. Acta* **2006**, *1762*, 124−130.
(35) Garsetti, M.; Pellegrini, N.; Baggio, C.; Brighenti, F. Antioxidant activity in human faeces. *Br. J. Nutr.* **2000**, *84*, 705−710.
(36) Tuohy, K. M.; Probert, H. M.; Smejkal, C. W.; Gibson, G. R. Using probiotics and prebiotics to improve gut health. *Drug Discovery Today* **2003**, *8*, 692−700.
(37) Kolida, S; Tuohy, K. M.; Gibson, G. R. Prebiotic effects of inulin and oligofructose. *Br. J. Nutr.* **2002**, *87*, 193−197.
(38) Gibson, G. R.; Wang, X. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. *J. Appl. Bacteriol.* **1994**, *77*, 412−420.
(39) Burns, A. J.; Rowland, I. R. Anti-carcinogenicity of probiotics and prebiotics. *Curr. Issues Intest. Microbiol.* **2000**, *1*, 13−24.
(40) Gibson, G. R.; Beaumont, A. An overview of human colonic bacteriology in health and diseases. In *Gut Flora and Health, past, present and future*; Leeds, A. R., Rowland, I. R., Eds.; The Royal Society of Medicine Press: London, 1996; pp 3−11.
(41) Ranganna, K. F.; Yatsu, F. M.; Hayes, B. E.; Milton, S. G.; Jayakumar, A. Butyrate inhibits proliferation-induced cell nuclear antigen expression (PCNA) in rat vascular smooth muscle cells. *J. Clin. Invest.* **2000**, *102*, 910−918.
(42) Cavaglieri, C. R.; Nishiyama, A.; Fernandez, L. C.; Curi, R.; Miles, E. A.; Calder, P. C. Differential effects of short chain fatty acids on proliferation and production of pro- and anti-inflammatory citokines by cultured lymphocytes. *Life Sci.* **2003**, *73*, 1683−1690.
(43) Lupton, J. R. Microbial degradation products influence colon cancer risk: the butyrate controversy. *J. Nutr.* **2004**, *134*, 479−482.
(44) Altonsy, M. O.; Andrews, S. C.; Tuohy, K. M. Differential induction of apoptosis in human colonic carcinoma cells (Caco-2) by Atopobium, and commensal, probiotic and enteropathogenic bacteria: Mediation by the mitochondrial pathway. *Int. J. Food Microbiol.* **2010**, *137*, 190−203.
(45) Hughes, R.; Magee, E. A. M.; Bingham, S. Protein degradation in the large intestine: relevance to colorectal cancer. *Curr. Issues Intest. Microbiol.* **2000**, *1*, 51−58.
(46) Lee, H. C.; Jenner, A. M.; Low, C. S.; Lee, Y. K. Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Res. Microbiol.* **2006**, *157*, 876−884.
(47) Puupponen-Pimia, R.; Nohynek, L.; Hartmann-Schmidlin, S.; Kahkonen, M.; Heinonen, M.; Maatta-Riihinen, K.; Oksman-Caldentey, K. M. Berry phenolics selectively inhibit the growth of intestinal pathogens. *J. Appl. Microbiol.* **2005**, *98*, 991−1000.
(48) Rodriguez-Vaquero, M. J.; Alberto, M. R.; Manca de Nadra, M. C. Antibacterial effect of phenolic compounds from different wines. *Food Control* **2007**, *18*, 93−101.
(49) Nohynek, L. J.; Alakomi, H. L.; Kähkönen, M. P.; Heinonen, M.; Helander, I. M.; Oksman-Caldentey, K. M.; Puupponen-Pimiä, R. H. Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens. *Nutr. Cancer* **2006**, *54*, 18−32.
(50) Molan, A. L.; Liu, Z.; Kruger, M. The ability of blackcurrant extracts to possitively modulate key markers of gastrointestinal function in rats. *World J. Microbiol. Biotechnol.* **2010**, *26*, 1735−1743.
(51) Bialonska, D.; Ramnani, P.; Kasimsetty, S. G.; Muntha, K. R.; Gibson, G. R.; Ferreira, D. The influence of pomegranate by-product and punicalagins on selected groups of human intestinal microbiota. *Int. J. Food Microbiol.* **2010**, *140*, 175−182.
(52) Tzounis, X.; Vulevic, J.; Kuhnle, G. G. C.; George, T.; Leonczak, J.; Gibson, G. R.; Kwik-Uribe, C.; Spencer, J. P. E. Flavonol monomer-induced changes to the human faecal microbiota. *Br. J. Nutr.* **2008**, *99*, 782−792.