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Protective Effect of an Avocado Peel Polyphenolic Extract Rich in Proanthocyanidins on the Alterations of Colonic Homeostasis Induced by a High-Protein Diet

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Supporting Information

ABSTRACT: Avocado peel, a byproduct from the avocado pulp industry, is a promising source of polyphenolic compounds. We evaluated the effect of a proanthocyanidin-rich avocado peel polyphenol extract (AvPPE) on the composition and metabolic activity of human fecal microbiota cultured for 24 h in a bioreactor in the presence of high protein (HP) levels and the effect of the resulting culture supernatants (CSs) on HT-29Glc$^{-/+}$ and Caco-2 cells. AvPPE decreased the HP-induced production of ammonia, H_2S, propionate, and isovalerate and increased that of indole and butyrate. Microbiota composition was marginally affected by HP, while AvPPE increased Actinobacteria, Coriobacteriaceae, Ruminococcaceae, and *Faecalibacterium*. AvPPE failed to prevent the HP-induced decrease of HT-29Glc$^{-/+}$ cell viability and energy efficiency but prevented the HP-induced alterations of barrier function in Caco-2 cells. Additionally, the genotoxic effect of the CSs upon HT-29Glc$^{-/+}$ was attenuated by AvPPE. Therefore, AvPPE may be considered as a promising product for improving colonic homeostasis.

KEYWORDS: *proanthocyanidins, high protein, protein metabolites, bioreactor, intestinal microbiota*

INTRODUCTION

Avocado (*Persea americana* Mill.) is a fruit of high nutritional quality, which contains high levels of unsaturated fatty acids, vitamins, minerals, proteins, and fibers.[1] About five million tons of avocados are produced yearly in the world, with peel and seeds generated in large amounts by the avocado pulp industry.[2] These agrofood byproducts are currently considered as promising sources of polyphenolic compounds including phenolic acids and flavonoids.[3,4] Dietary polyphenols are nonessential nutrients that exert a wide range of biological activities and health-promoting effects in humans.[5] The regular intake of polyphenol-containing foodstuffs is associated with a lower risk and severity of noncommunicable chronic diseases.[5] Flavan-3-ols (or flavanols) are the most complex subclass of flavonoids that includes from simple monomers to high-molecular-weight polymeric proanthocyanidins (PACs).[6] Typically, PACs could be catechin and epicatechin condensation products with a degree of polymerization ranging from 3 to 30 units in oligomeric fractions or >30 in polymeric fractions. PACs are present in many foodstuffs and beverages like seeds, fruits, red wine, cider, tea, cocoa, and beer.[6,7] Only PACs with a degree of polymerization ≤3 are absorbed in the small intestine. The oligomeric and polymeric forms, however, accumulate in the lumen and reach the colon where they are metabolized by the intestinal microbiota (IM), releasing numerous metabolites that may be absorbed by the colonic epithelium and pass to the bloodstream.[6] In the colon, PACs can also modulate decomposition of the IM exerting prebiotic activities, stimulating the growth of *Lactobacillus* spp. and *Bifidobacterium* spp. as well as some butyrate-producing bacteria.[6] Thus, the gastrointestinal tract constitutes a key organ for the health-promoting effects of dietary PACs and it is probable that part of their beneficial impact at the extraintestinal level can be attributed to their circulating, low-molecular-weight, microbiota-derived metabolites.[6] We recently reported that PAC-rich polyphenol extracts from grapes, cranberry, apple, and avocado, as well as PAC-derived

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bacterial metabolites (3-phenylpropionic acid, 3,4-dihydroxyphenyl propionic acid, and 4-hydroxyphenyl acetic acid) prevented the deleterious effects of the microbiota-derived protein metabolites, *p*-cresol and H_2S, on HT-29Glc$^{-/+}$ and Caco-2 cells, suggesting that dietary PACs might protect against the negative impact of high-protein diets (HPDs) on colonic epithelial cells.[8,9]

High-protein diets (HPDs) bring 25−35% of their energy as proteins, compared with normo-protein diets that only contribute with 12−18% of the energy intake.[10] They are widely used by athletes for increasing their muscle mass and by overweight and obese subjects for losing weight, through their ability to stimulate satiety and increase thermogenesis.[10,11] About 90% of the dietary proteins are digested and absorbed in the small intestine, and the remaining 10% reach the colon undigested or partially digested. Accordingly, a higher intake of dietary proteins generally means a higher flux of undigested proteins reaching the colon.[12] In the colon, undigested proteins are hydrolyzed by proteases from the IM. The released amino acids are used for bacterial protein synthesis and growth or are fermented, generating an array of bacterial metabolites that accumulate in the colonic lumen.[13,14] While short-chain fatty acids (SCFA) are considered as beneficial metabolites,[14,15] other byproducts including ammonia (NH_3), hydrogen sulfide (H_2S), phenol, *p*-cresol, and polyamines at excessive concentrations are potentially deleterious for the colonic epithelium and at the systemic level.[12−14] In the colon, proteins can also modulate the composition of the intestinal microbiota, favoring the development of pathogenic and pro-inflammatory populations like *Clostridium perfringens*, *Enterococcus*, *Shigella*, and *Escherichia coli* spp. and diminishing those beneficial like *Bifidobacterium* spp.[13] The colonic fermentation of proteins by the IM is, therefore, considered as a potentially harmful process for host health, which could enhance the risk of diseases such as colorectal cancer and ulcerative colitis.[12−14]

Accordingly, the aim of this study was to evaluate the preventive effect of an avocado peel polyphenolic extract rich in proanthocyanidins on the changes in human fecal microbiota cultured in the presence of high protein amounts and on the decrease in the production of protein-derived bacterial metabolites and their detrimental effect on colonic epithelial cells.

MATERIALS AND METHODS

Avocado Peel Polyphenol Extract (AvPPE). Avocado peels were obtained from fresh avocado fruits (*P. americana* Mill. Var. Hass) purchased from local market, and AvPPE was prepared by the Laboratory of Pharmacognosy at the University of Concepción (Concepción, Biobío, Chile) and subsequently freeze-dried and stored at −70 °C until use. The elaboration of the extract and the characterization of its polyphenol composition have been previously described.[8] Thirty seven grams of fresh avocado peel was necessary to obtain 1 g of AvPPE.[16] Briefly, frozen samples of avocado peels (500 g) were mixed with hot water (90 °C, 5 min) and then filtered (sintered glass funnel, 70−100 μm). Marcs were homogenized and extracted again with hot water (65 °C). After maceration (60 min), sugars and water-soluble compounds were removed with distilled water by the use of a glass column packed with Sepabeads SP-850 (Supelco, Bellefonte). Polyphenol extract was obtained by the same procedure but eluted with absolute ethanol and then was evaporated under vacuum, freeze-dried, and stored at −70 °C until use.[8] The identification and characterization of polyphenolic extract was carried out by RP-HPLC in a Waters Alliance 2695 system equipped with a C18 column and C18 pre-column. Quantification was carried out using a UV−vis chromatogram. Standards were used to perform calibration curves.[8] AvPPE contained 14% of flavan-3-ol monomers, 29.1% of proanthocyanidins (PACs, type A and B, including only epicatechin monomers, with a mean degree of polymerization of 6.1), 15.1% of other flavonoids, 3% of anthocyanins, and 38.8% of phenolic acids, as shown in Table S1.

Donor Recruitment. This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee for Research in Humans, Faculty of Medicine, University of Chile (No. 118-2014). Healthy subjects recruited in the study must provide a written informed consent. Smokers and subjects with intake of antibiotics or probiotics in the last 2 months before stool collection were excluded from the study. Subjects were selected according to the presence of sulfate-reducing bacteria (SRB) in their microbiota, due to the fact that these microorganisms produce H_2S by coupling oxidative phosphorylation with sulfate reduction[17] and that H_2S was one of the protein bacterial metabolites determined in our study. Subjects were considered as SRB-positive based on the polymerase chain reaction (PCR) amplification of the *aprA* gene that encodes the α-subunit of the adenosine-5′-phosphosulfate reductase present in all known SRB.[18] Fecal bacterial DNA coming from a single stool sample was extracted by the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) using the recommendations of the manufacturer, with an additional step of mechanic lysis with a bead beater. PCR was carried out using the *aprA* gene primers Fwd.: 5′-TGGCAGAT-MATGATYMACGG-3′ and Rev.: 5′-GGGCCGTAACCGTCCTT-GAA-3′.[18] DNA samples from *Desulfovibrio piger* and *Desulfovibrio piezophilus* (DSMZ, Braunschweig, Germany) and from *C. perfringens* and *E. coli* were used as positive and negative controls, respectively. Quantification of SRB in PCR-positive subjects was performed by qPCR using a LightCycler 2.0 (Roche, Basel, Switzerland) and the LightCycler FastStart DNA Master SYBR Green I kit (Roche, Basel, Switzerland); standard curves were carried out with *Dromaeolus piger* DNA.

Fecal Sampling. Five days before and during fecal sampling, the recruited subjects were counseled by a registered dietitian to consume a standardized diet (1800 and 2000 kcal/day for women and men, respectively; 15% proteins, 58% carbohydrates, and 27% fat), with the aim of decreasing the inter- and intraindividual variability in their microbiota composition. Stools were collected in sterile plastic recipients, maintained in anaerobic conditions (GasPak, Becton-Dickinson, New Jersey), and refrigerated (4 °C) until processing, within the 3 h following their emission. Every 2 days, each recruited subject provided one fresh stool sample, until four samples were provided. Each sample was used for one independent assay.

In Vitro Fermentation of Human Fecal Microbiota. Fecal inocula and bioreactor cultures were performed in a pre-reduced culture medium containing peptone (2 g/L), yeast extract (2 g/L), NaCl (100 mg/L), K_2HPO_4 (40 mg/L), KH_2PO_4 (40 mg/L), $NaHCO_3$ (2 g/L), $MgSO_4{\cdot}7H_2O$ (10 mg/L), $CaCl_2{\cdot}2H_2O$ (10 mg/L), tween 80 (2 mL/L), hemin (50 mg/L), vitamin K1 (10 μL/L), bile salts (500 mg/L), resazurin (1 mg/L), and glucose (10 g/L) in distilled and sterile water as previously described,[19] with minor modifications. The sterile culture medium was gassed for 1.5 h with N_2 before inoculation. For the inoculum preparation, fresh stools were diluted in culture medium at a concentration of 100 g/L and then were mixed in a stomacher for 30 s. After that, the mix was centrifuged at 450*g* for 2 min. The supernatant of this centrifugation was inoculated directly in the culture medium of the bioreactor at a final concentration of 1% of stools. Depending on the treatment, the culture medium was used alone (control (C)) or supplemented with 1.2 g/L AvPPE (AvPPE), 2 g/L proteins (hyperproteic (HP)), or 2 g/L proteins and 1.2 g/L AvPPE (HP/AvPPE). The protein was a mix of casein (80%) (Sigma, Missouri) and whey protein (20%) (Hilmar, California). Culture was conducted for 24 h at 37 °C in anaerobic conditions under N_2 flow and constant agitation, using a Micro DCU-200 bioreactor system (B. Braun Biotech International, Berlin, Germany). pH was automatically maintained at 6.8 by injecting HCl (0.5 M) or NaOH (1 M). Samples of culture supernatants (CSs) were collected at 1 h (T1) and 24 h (T2) after

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inoculation and immediately aliquoted and frozen at −80 °C until analysis.

Determination of Protein Metabolites. *Ammonia (NH_3).* Thawed CSs were centrifuged two times successively (15 min, 3405*g*, 4 °C), and the supernatants were filtered (0.22 μm) and deproteinized (10 kDa Microcon centrifugal filter devices) (Merck, Santiago, Chile). The determination of NH_3 was carried out in 96-well plates using a commercial assay kit (K-AMIAR, Megazyme, Illinois) based on the reaction of ammonia (as ammonium ions; NH_4^+) with 2-oxoglutarate to form L-glutamic acid and $NADP^+$ in the presence of glutamate dehydrogenase and nicotinamide adenine dinucleotide phosphate (NADPH). The decrease in absorbance at 340 nm, due to NADPH oxidation, was registered as a reflection of the ammonia concentration in the sample.

Hydrogen Sulfide (H_2S). H_2S determination was carried out by gas chromatography−mass spectrometry (GC−MS) after sulfide alkylation, as previously described.[20,21] Thawed CSs were vortexed and centrifuged (14 000*g*, 5 min, 4 °C); 100 μL of supernatant was placed in a vial with 100 μL of 5 mM benzalkonium chloride (prepared in deoxygenated MiliQ water saturated with sodium tetraborate), 100 μL of 20 mM pentafluorobenzylbromide (in toluene), and 100 μL of ethyl acetate (containing 100 μM naphthalene as internal standard); and shacked at 55 °C for 4 h in a rotating shaker. Thereafter, 150 μL of KH_2PO_4 (saturated in MiliQ water) was added, the mixture was centrifuged (10 000*g*, 10 min, 4 °C), and the organic phase was removed. One microliter was injected in an Agilent 6890N gas chromatograph equipped with an HP-5HS capillary column (using helium as gas carrier) and a 5973N MS detector (Agilent Technologies, California). Calibration curves were made with standard Na_2S solutions (as donor of H_2S) between 12.5 and 500 μM. The temperature of the injector and the transfer line was maintained at 250 °C and those of the ion source and the quadrupole detector were 230 and 150 °C, respectively. The GC program was as follows: 70 °C for 1 min, 100−180 °C, ramp 8 °C/min, 180−300 °C, ramp 50 °C/min, maintained for 2 min. The areas under the curves of the samples and standard solutions ((bis-(pentafluorobenzyl) sulfide (a derivate of sulfide)) were corrected by the area under the curve of the internal standard, and the results were expressed as μM.

Indole. Thawed CSs were vortexed and centrifuged (10 min, 15 805*g*, room temperature). The supernatant was diluted four times with 70% ethanol, and 100 μL was used for indole determination as previously described.[22] A calibration curve was made with indole standards prepared in 70% ethanol, in a range of concentration between 0 and 300 μM. For spectrophotometric determination, 100 μL of sample or standards were mixed in a 96-well plate with 25 μL of 5.3 M NaOH and 50 μL of 0.3 M hydroxylamine hydrochloride (NH_2OH-HCl). After 15 min of incubation, 125 μL of 2.7 M H_2SO_4 was added and the mixture was vortexed and incubated for 30 min at room temperature. Absorbance was measured at 530 nm in an Infinite 200 PRO plate reader (TECAN, Männedorf, Switzerland).

Short-Chain Fatty Acids (SCFAs). SCFA determination was carried out as previously described[23] with some modifications. The pH of the thawed CSs was adjusted to 2−3 with 0.68 M HCl. After incubation for 10 min at room temperature with occasional agitation, the samples were centrifuged (10 min, 15 805*g*) and 2-ethyl butyric acid, as internal standard, was added at a final concentration of 1 mM. The detection and quantification of SCFAs were performed in an Agilent 7890A gas chromatograph (Agilent Technologies, California) equipped with an FID detector and a Restek Stabilwax-Da (fused silica) capillary column (Restek, Pensilvania) (30 m length, 0.32 mm i.d.). The separation of the SCFAs was carried out between 120 and 240 °C, with an initial heating of 120 °C for 1 min, followed by an increase to 240 °C at a rate of 10 °C/min, and then held for 3 min at 240 °C. Hydrogen was used as a carrier gas, and the temperature of the injector and detector was 240 and 265 °C, respectively. A mix of acetic acid, propionic acid, butyric acid, valeric acid, isobutyric acid, and isovaleric acid (1 mg/mL) (Restek, Pensilvania) was used for calibration. The area under the curve of the samples and standard solutions was corrected by that of the internal standard.

Determination of the Composition of Microbiota by MiSeq Sequencing. Bacterial genomic DNA was purified from the CSs using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) with a supplementary step of mechanic lysis with a bead beater. The composition of microbiota was analyzed by MiSeq sequencing in the Roy J. Carver Biotechnology Center (University of Illinois, Illinois) following a methodology previously described.[24] Briefly, the region V3 and V4 of the 16S rRNA gene was amplified by PCR and subsequently the amplicons generated were sequenced using the Illumina MiSeq platform. The sequencing service of the W.M. Keck Center for Comparative and Functional Genomics (University of Illinois, Illinois) delivered demultiplexes and barcode depleted sequences. Paired-end sequences were joined using PEAR, and primer sequences were depleted by fastx-trimmer from FASTX-Toolkit. QIIME software (V1.9.1) was used to analyze the 16S rRNA gene sequences.[25] Using open-reference command, operational taxonomic units (OTUs) were selected and defined by clustering at 3% divergence (97% similarity) using the GreenGenes database as reference.[26] Results were expressed as relative abundance.

The raw data paired-end reads obtained from the MiSeq platform were stored in the ENA online public database under accession number PRJEB30984 (http://www.ebi.ac.uk/ena/data/view/PRJEB30984).

Effect of CSs on Cell Viability, Mitochondrial Function, Barrier Function, and Genotoxic Damage in Intestinal Cell Lines. *Cell Viability.* Thawed CSs (T2, 24 h) were centrifuged (15 min, 3405*g*, 4 °C), and the supernatants of this centrifugation were centrifuged again (15 min, 3405*g*, 4 °C) and then subsequently filtered (0.22 μm). The effect of CS incubation on cell viability was assayed in HT-29Glc$^{-/+}$ cells, as previously described.[8] Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Corning, New York), 10% heat-inactivated fetal calf serum (Gibco, ThermoFisher Scientific, Massachusetts), and 1% penicillin/streptomycin (Hyclone, ThermoFisher Scientific, Massachusetts). At day 0, cells were seeded in 96-well plates at a density of 2×10^4 cells/cm^2. At day 3, the medium was changed, and from day 4 to 6, cells were treated with the CSs at a final concentration of 10%, with daily change of the culture medium. At day 7, cell viability was measured through the quantification of the lactate dehydrogenase (LDH) (CytoTox One Kit, Promega, Wisconsin) released in the culture medium. LDH is a cytosolic enzyme that can be released to the culture medium when there is a loss in the cellular membrane integrity, as reflex of loss in cell viability. Thus, LDH quantification in the culture medium is an indirect way to measure cell viability.[27] The results were normalized expressing the value of LDH released in the culture medium as a function of total LDH of the cells. To that, cells that remained in the plate after the quantification of LDH release were lysed with Triton 0.02% to quantify the total LDH present in cells.

Mitochondrial Function. The effect of CSs on HT-29Glc$^{-/+}$ energy metabolism was evaluated by measuring the mitochondrial function by polarography using an Oxygraph Strathkelvin 782 2-channel oxygen system (Strathkelvin Instruments, North Lanarkshire, Scotland) equipped with a 1302-type Clark electrode (Strathkelvin Instruments, North Lanarkshire, Scotland) with a polypropylene membrane, as previously described[28,29] with some modifications. HT-29Glc$^{-/+}$ cells were cultured as previously described (Cell Viability section). At day 0, cells were seeded in 24-well plates at a density of 2×10^4 cells/cm^2. At day 3, the medium was changed, and from day 4 to 6, cells were treated with the CSs at a final concentration of 10%, with daily change of the culture medium. At day 7, cells were trypsinized and put into the oxygraph chamber at a density of 750 000 cells/mL in respiration medium (20 mM HEPES, 200 mM mannitol, 5 mM KH_2PO_4, 2.5 mM $MgCl_2$, 0.5 mM EGTA, 25 mM glucose, 2 mM L-glutamine, pH 7.4, enriched with bovine serum albumin 0.1%), without cell culture medium and treatments. The oxygen consumption rate (OCR, nmol O_2/min/10^6 cells) was evaluated at baseline, after the addition of 2 μM oligomycin (an inhibitor of adenosine 5′-triphosphate (ATP) synthase) to quantify the oxygen consumption due to the proton leak (i.e., not coupled to ATP

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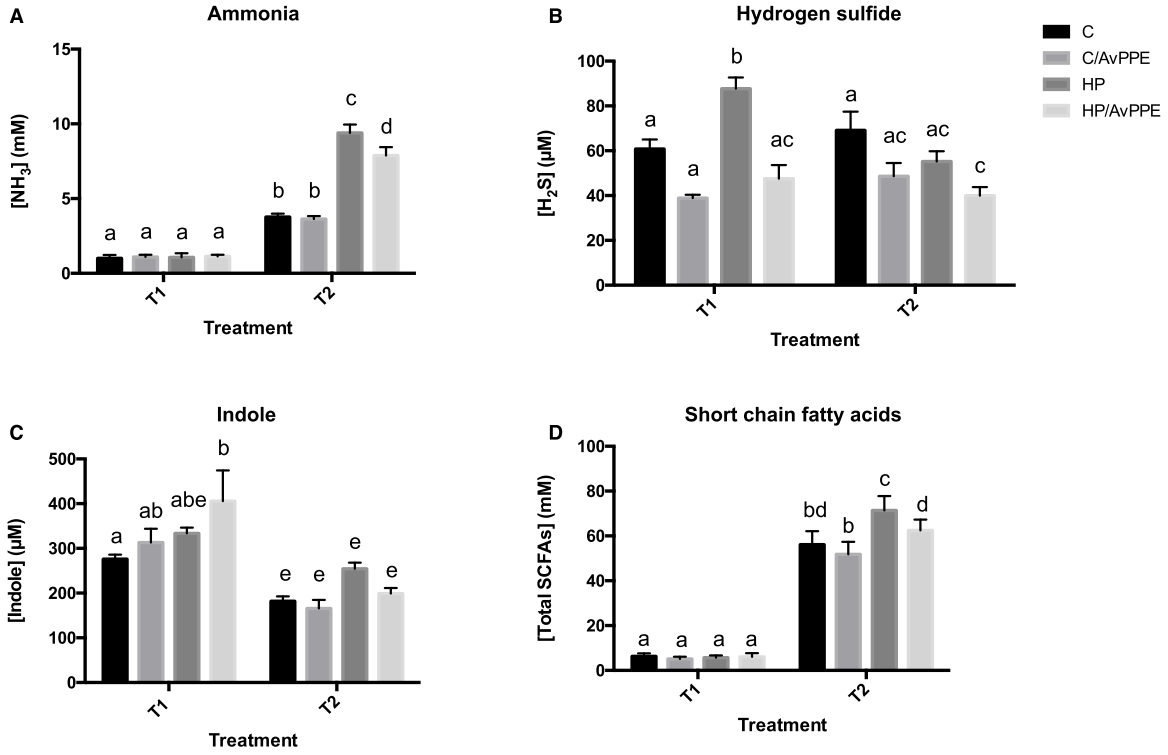

Figure 1. Concentrations of NH_3 (A), H_2S (B), indole (C), and total SCFAs (D) in the CSs after 1 (T1) and 24 h (T2) of fermentation. Each bar represents the average of five subjects (with SEM). Data were analyzed by two-way ANOVA with repeated measures (time effect: $p = 0.0002$ (A), $p = 0.21$ (B), $p = 0.0019$ (C), and $p = 0.0004$ (D); treatment effect: $p < 0.0001$ (A), $p < 0.0001$ (B), $p = 0.10$ (C), and $p = 0.0079$ (D); interaction time × treatment: $p < 0.0001$ (A), $p = 0.0105$ (B), $p = 0.0580$ (C), and $p = 0.0022$ (D)). Bars with different letters are significantly different ($p < 0.05$).

synthesis), and after the addition of 3 μM FCCP (a mitochondrial inner membrane uncoupler that dissipates the mitochondrial membrane potential and, in consequence, accelerates electron flux through the electron transport chain) to quantify maximal OCR. The ATP production linked to OCR (as the difference between basal OCR and proton leak) and the reserve capacity (as the difference between the maximal and basal OCRs) were calculated.[28,29] All of these parameters were expressed as % of basal OCR.

Intestinal Barrier Function. The effect of CSs on the intestinal barrier function was determined in Caco-2 cells (ATCC, Virginia) by measuring changes in transepithelial electrical resistance (TEER). Cells (passages 10−21) were cultured in DMEM/Ham's F12 medium supplemented with L-glutamine (Corning, New York), 10% heat-inactivated fetal calf serum (Gibco, Thermo Fisher Scientific, Massachusetts), 1% penicillin/streptomycin (Hyclone, ThermoFisher Scientific, Massachusetts), and 1% nonessential amino acid solution (Corning, New York), as previously described.[8] At day 0, cells were seeded at a density of 10^5 cells/cm^2 in Transwell polycarbonate filters (12 mm diameter, 0.4 μm pore size) (Corning-Costar, New York) previously treated with 0.044 μg/μL collagen in 0.01 M HCl. The apical and basolateral compartments of the filters were filled with 500 and 1000 μL of culture medium, respectively, and the culture medium was changed three times per week. Once the TEER reached 1000 Ω cm^2 (approximately 6−7 days after seeding), 30% of the apical culture medium was replaced with the CSs. TEER was measured with a milliohm meter (EVOM, World Precision Instruments, Florida) at baseline and 24 h after medium replacement.

Genotoxic Damage. The genotoxic damage induced by the CSs in HT-29Glc$^{-/+}$ cells was assessed through the quantification of phosphorylated histones using the H2A.X phosphorylation assay kit (Merck, Santiago, Chile). H2A.X histones phosphorylate in response to the breakdown of the double strand of DNA. HT-29Glc$^{-/+}$ cells were cultured as mentioned above (Cell Viability section) at a density of 36 × 10^3 cells/well in 96-well plates as previously described.[29] After 16 h, complete culture medium was replaced with culture medium without fetal calf serum and with 15% CS, and after 24 h, the levels of genotoxic damage were evaluated by quantifying phosphorylated H2A.X histones by luminescence. Results were expressed as the % of the positive control (etoposide 10 μM).

Statistical Analysis. Statistical analysis was carried out with GraphPad Prism, version 6.0. Variables were expressed as mean and standard error of the mean (SEM). The comparisons between the means in each assay were performed by two-way analysis of variance (ANOVA) with repeated measures. For variables measured only at 24 h of fermentation, the analyses were performed by one-way ANOVA with repeated measures and Tukey's post-hoc test (if the distribution of variables was normal, assessed by the Shapiro−Wilk test) or by Friedman's test and Dunn's post-hoc test (if the distribution of variables was not normal). Correlations between the different parameters (metabolites and bacterial taxa) were carried out using Spearman's rank correlation coefficient. The statistical significance was considered with $p < 0.05$.

RESULTS

Donor Recruitment. Eight out of twelve subjects evaluated (66.7%) were positive for the presence of SRB in their stools, assessed by PCR. After a qPCR quantification of SRB, we selected the subject participants based on their similitude in their counts, which ranged between 1.05 × 10^6 and 5.82 × 10^8 copies of *aprA* gene/g stool. We selected five subjects who had an average of 7.02 × 10^7 copies of *aprA* gene/g stool. They were aged 26−37 years (mean ± standard deviation: 31.6 ± 4.5 years), had a normal weight (body mass index: 23.3 ± 2 kg/m^2), and were healthy and omnivores.

Determination of Protein Metabolites in Culture Supernatants (CSs). No differences in NH_3 concentration between treatments were observed at 1 h of fermentation (T1) (Figure 1A). At 24 h (T2), NH_3 concentration significantly

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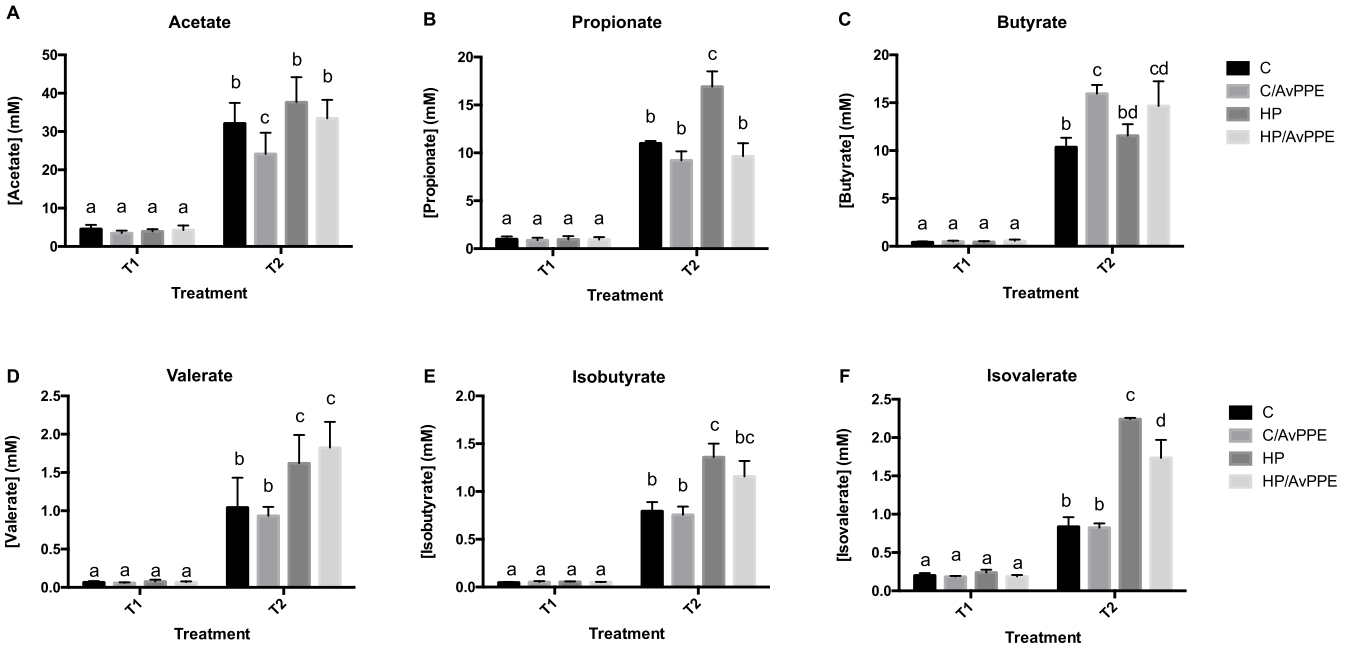

Figure 2. Concentration of uSCFAs (acetate (A), propionate (B), butyrate (C), and valerate (D)) and bSCFAs (isobutyrate (E) and isovalerate (F)) in the CSs at 1 (T1) and 24 h (T2) of fermentation. Each bar represents the average of five subjects (with SEM). Data were analyzed by two-way ANOVA with repeated measures (time: $p = 0.0054$ (A), $p < 0.0001$ (B), $p = 0.0002$ (C), $p = 0.0112$ (D), $p < 0.0001$ (E), $p = 0.0001$ (F); treatment: $p = 0.0046$ (A), $p = 0.0044$ (B), $p = 0.037$ (C), $p = 0.0068$ (D), $p = 0.0337$ (E), $p < 0.0001$ (F); interaction time × treatment: $p = 0.012$ (A), $p = 0.0023$ (B), $p = 0.039$ (C), $p = 0.0060$ (D), $p = 0.0275$ (E), $p < 0.0001$ (F)). Bars with different letters are significantly different ($p < 0.05$).

increased in all of the treatments, compared with T1. Additionally, the concentration of NH_3 significantly increased with HP compared with C, and this increase was partially prevented in HP/AvPPE.

H_2S concentration at T1 was higher with HP than with C and C/AvPPE, and this increase was prevented with the HP/AvPPE treatment (Figure 1B). Only HP treatment showed variations in H_2S concentration between T1 and T2, in which a significant decrease was observed. The concentration of H_2S at T2 was significantly lower with HP/AvPPE than with C.

Indole concentration at T1 (Figure 1C) significantly increased with HP/AvPPE compared with C. At T2, indole concentration decreased significantly except with HP; no differences between the four treatments were detected at T2.

SCFA concentrations were low at T1, without differences between treatments, and significantly increased at T2 for all conditions (Figure 1D). This increment was due to a significant increase in both unbranched (uSCFAs) and branched short-chain fatty acids (bSCFAs) (Figure S1). It is noteworthy that the concentrations of total SCFAs significantly increased with HP compared with C and C/AvPPE, and this increase was partially prevented with HP/AvPPE; moreover, the concentration of bSCFA strongly increased with HP and HP/AvPPE, compared with C and AvPPE.

When evaluating each SCFA individually (Figure 2), acetate, propionate, and butyrate were the most abundant. All SCFAs increased significantly at T2 compared with T1 and with all of the treatments. At T2, acetate significantly decreased and butyrate increased in C/AvPPE compared with C (Figure 2A,C). HP induced a significant increase in the production of propionate, valerate, and the bSCFAs isobutyrate and isovalerate (Figure 2B,D–F). The increase of propionate and isovalerate was prevented with HP/AvPPE (Figure 2B,F).

Effect of the Treatments on the Microbiota Composition. A total of 1 874 289 sequences were obtained after trimming, assembly, quality filtering, and chimera checking, which exhibited an average of 455 ± 9.8 bp sequence length. After OTU assignment, a total of 1 504 917 counts were obtained in BIOM table, ranging from 18 853 to 94 706 counts per sample. The rarefaction curves reached an asymptote (Figure S2A), indicating that the number of sequences obtained per sample was sufficient to describe the diversity of the bacterial communities of the samples, without significant differences between them. Considering the taxa with a relative abundance >0.1%, 8 phyla, 22 families, and 32 genera were identified. No differences between the four treatments were detected in phylum, family, or genus at T1. The core microbiota, which represents the genera present in all of the subjects in the control condition at T1, represented the 29.8% of the total microorganisms (Table S2). The intraindividual α diversity, evaluated through the Chao1 index, did not show any significant differences between the four treatments at T2 ($p = 0.52$). The inter-individual β diversity, evaluated at T2 through weighted Unifrac, did not show significant differences (Figure S2B). The prevalence and relative abundance of the bacterial phyla, families, and genera at T2 for each of the four treatments are shown in Tables S3–S5, respectively.

Considering the average of the four treatments at T2, the phyla Firmicutes (42%), Bacteroidetes (34.8%), Proteobacteria (22.2%), and Actinobacteria (0.44%) were the most abundant, while the others, globally, had a relative abundance of 0.15%. The relative abundance of the Actinobacteria phylum at T2 significantly differed between the treatments, increasing with HP/AvPPE compared with C (Figure 3A). At the family level, considering the average of the four treatments at T2, Bacteroidaceae (21.7%) was the most abundant, followed by Lachnospiraceae (12.3%), Enterobacteriaceae (11.2%), Ruminococcaceae (10.3%), and Porphyromonadaceae (6.92%). The percentage of microorganisms not identified was 6.5%. The other families as a whole had a relative abundance of 22.7%. At

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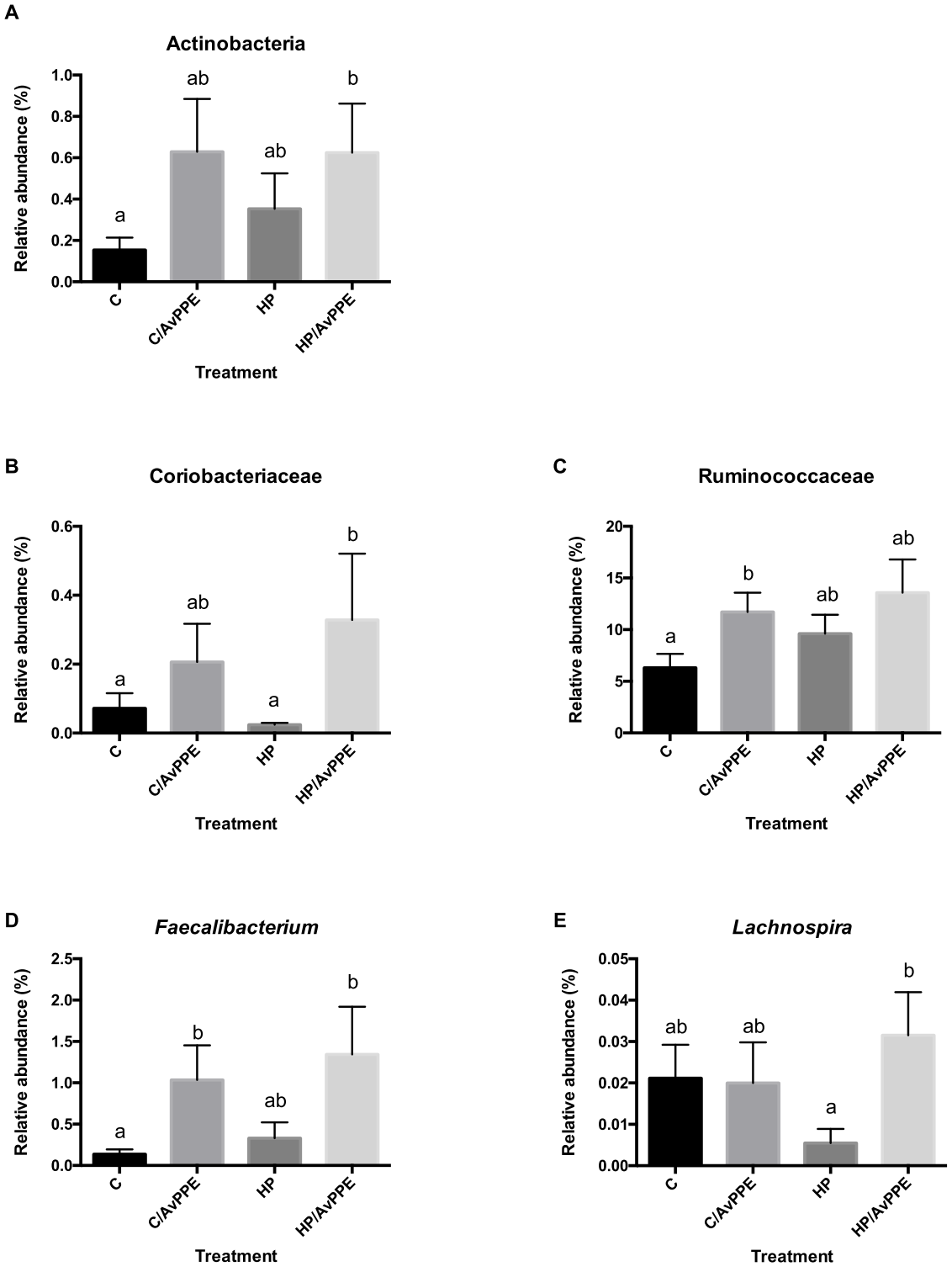

Figure 3. Relative abundance (%) of phyla, families, and genera of CSs that showed significant differences at T2. Each bar represents the average of five subjects (with SEM). Data were analyzed by Friedman's test with repeated measures ((A) $p = 0.02$; (B) $p = 0.006$; (C) $p = 0.02$; (D) $p < 0.001$; (E) $p = 0.008$) and Dunn's post-hoc test. Bars with different letter are significantly different ($p < 0.05$).

T2, two families, Coriobacteriaceae and Ruminococcaceae, exhibited significant differences between treatments. The relative abundance of Coriobacteriaceae increased with HP/AvPPE, compared with C and with HP. The relative abundance of Ruminococcaceae increased significantly with C/AvPPE and not significantly ($p = 0.08$) with HP/AvPPE, compared with C (Figure 3B,C).

At the genus level, considering the average of the four treatments at T2, 33.9% of the microorganisms were not identified. *Bacteroides* (21.7%) was the most abundant genus, followed by *Parabacteroides* (6.9%), *Sutterella* (5.5%), [*Ruminococcus*] (4.4%), *Bilophila* (3.2%), [*Eubacterium*] (2.8%), and *Coprococcus* (2.6%). The other genera as a whole had a relative abundance of 8.3%. At T2, two genera,

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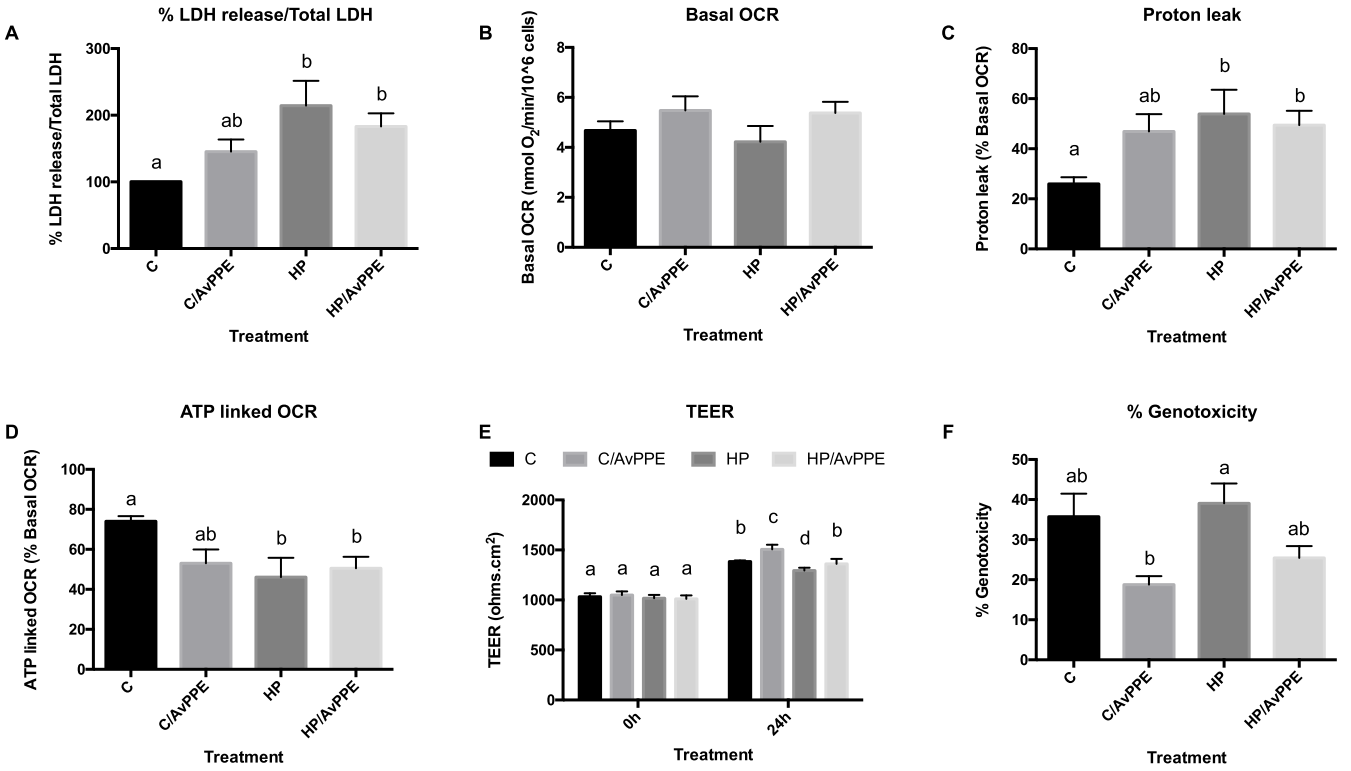

Figure 4. Effect of the CSs at T2 (10%, 72 h of incubation with daily replacement of the culture medium in HT-29Glc$^{-/+}$ cells) on the (A) LDH release* in regard to intracellular total LDH, (B) basal oxygen consumption rate (OCR), (C) proton leak, and (D) ATP-linked OCR. (E) effect of the CSs (30%, 24 h of incubation in Caco-2 cells) on the transepithelial electrical resistance, (F) effect of the CSs (15%, 24 h of incubation in HT-29Glc$^{-/+}$ cells) on the genotoxic damage**. Each bar represents the average of five subjects (with SEM), and each subject represents the average of three independent experiments (A–E) and two independent experiments (F). Data were analyzed by Friedman's test with repeated measures ((A) $p = 0.0055$; (B) $p = 0.65$; (C) $p = 0.012$; (D) $p = 0.012$; (F) $p = 0.0031$), Dunn's post-hoc test, and two-way ANOVA with repeated measures (E) (time: $p < 0.0001$; treatment: $p = 0.0001$; interaction time × treatment: $p = 0.0005$). Bars with different letters are significantly different ($p < 0.05$). * Control treatment was considered as 100% of LDH release. ** Expressed in % of the positive control etoposide (100% of genotoxicity).

Faecalibacterium and *Lachnospira*, showed significant differences between the treatments. *Faecalibacterium* increased significantly in C/AvPPE and HP/AvPPE, compared with C (Figure 3D), while the relative abundance of *Lachnospira* was lower with HP than with HP/AvPPE (Figure 3E).

Effect of CSs on Cell Viability, Mitochondrial Function, Barrier Function, and Genotoxic Damage in Intestinal Cell Lines. A significant increase of LDH release was observed with HP, compared with C. AvPPE alone did not affect the LDH release significantly, but it seems somewhat toxic for the cells. When AvPPE was combined with HP, it did not prevent the increase in LDH release (Figure 4A).

A basal OCR of 4.7 nmol O_2/min/10^6 cells was observed in C, this value being unaffected by the different treatments (Figure 4B). Proton leak, evaluated in the presence of oligomycin, represented 26% of the basal OCR with C. AvPPE alone seems to augment somewhat the proton leak (in a not significant way). Proton leak augmented significantly with HP. Such increase was not prevented with the presence of AvPPE (Figure 4C). Consequently, the ATP-linked OCR significantly decreased with HP and HP/AvPPE, compared with C (Figure 4D). Maximal OCR and cell reserve capacity were not affected with any treatments (data not shown).

Changes in the intestinal barrier function were evaluated by measuring TEER in Caco-2 cells grown in Transwell filters and exposed to CSs (Figure 4E). TEER was similar in the four treatments at the beginning of the incubation period (0 h) and increased significantly with all of the treatments after 24 h of exposition. At 24 h, TEER was significantly higher with C/AvPPE and lower with HP, compared with C. The decreased of TEER observed with HP was prevented with HP/AvPPE.

The genotoxic effect of CSs on HT-29Glc$^{-/+}$ cells was determined by H2A.X histone phosphorylation. As shown in Figure 4F, basal genotoxicity observed with C was 35.7% that of the positive control with etoposide. HP treatment has no effect on genotoxicity. In the C/AvPPE treatment, genotoxicity tended to be lower ($p = 0.08$) than with C and was significantly lower than with HP.

Correlations. Figure 5A shows the correlations observed between the different bacterial metabolites present in the CSs. Ammonia positively correlated with isovalerate, isobutyrate, valerate, and indole. Isovalerate positively correlated with propionate, acetate, isobutyrate, and indole.

In relation to bacterial genera, a positive correlation between hydrogen sulfide and *Bacteroides* was observed; acetate and *Bilophila*; propionate and *Coprococcus*; butyrate and *Sutterella*, *Prevotella*, *Desulfovibrio*, *Catenibacterium*, and *Bifidobacterium*; valerate and *Ruminococcus*; isobutyrate and *Sutterella*; and between isovalerate and *Sutterella* and *Butycirimonas*. On the other hand, a negative correlation was observed between hydrogen sulfide and *Faecalibacterium*, *Anaerostipes* and [*Prevotella*]; indole and *Lachnospira*; acetate and *Prevotella* and [*Ruminococcus*]; propionate and *Dorea* and [*Ruminococcus*]; valerate and *Bifidobacterium* and *Bacteroides*; and between isobutyrate and *Dorea* (Figure 5B).

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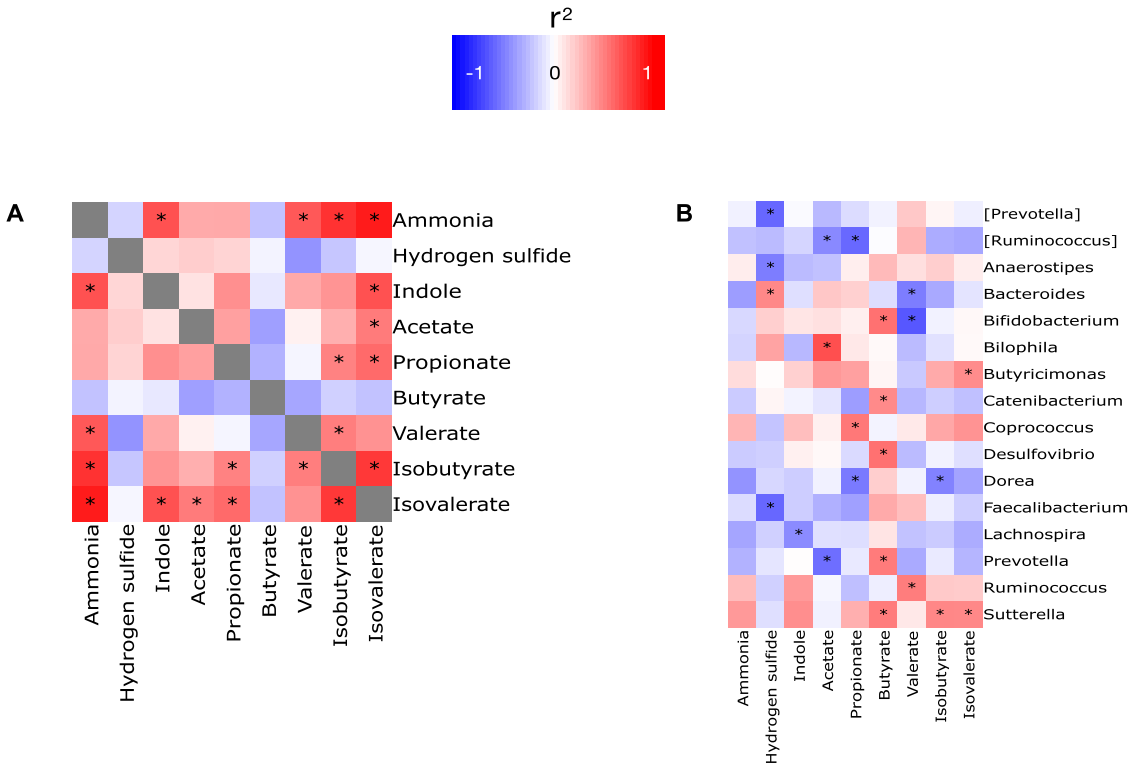

Figure 5. Heatmap of the correlations between the different parameters measured. All of the data were used for the analysis (all treatments for each parameter). The color indicates the value of the Spearman correlation coefficient. The asterisk indicates that the correlation is statistically significant ($p < 0.05$). (A) correlation between the metabolites of proteins. (B) correlation between the bacterial genera and metabolites of proteins.

DISCUSSION

The intestinal microbiota generates numerous metabolites from the undigested proteins that reach the colon, which can exert deleterious or beneficial effects at the colonic and, eventually, systemic levels. The first aim of this study was to quantify the in vitro production of NH_3, H_2S, indole, and SCFAs by human fecal microbiota in the presence of increased amount of proteins and to evaluate how the addition of AvPPE to the culture medium could affect this production. Our results showed that HP increased ammonia production, confirming previous observations that fecal and colonic ammonia levels depend on the amounts of ingested proteins.[14] The presence of high concentrations of ammonia in the colonic lumen has been shown to alter epithelium architecture, cellular energetic supply, mitochondrial oxygen consumption, SCFA oxidation capacity, and epithelial barrier function.[14] Accordingly, the fact that AvPPE partially prevented ammonia production can be considered as beneficial and is in agreement with a study that used condensed tannins from acacia and quebracho in an in vitro system with rumen microbiota.[30] In this study, as in our study, ammonia production decreased after 24 h of culture when tannins were added to the bioreactor.

One of the selection criteria of our subjects was the presence of SRB in their fecal microbiota, considering that these bacteria produce H_2S, one of the compounds determined in our study. SRB were detected in 66.7% of all subjects initially evaluated, which is within the range of reported values for these bacteria in human stools, both in prevalence and counts.[31] H_2S in excess is potentially toxic for the intestinal epithelium as it inhibits colonocyte respiration, increases colonocyte gene expression related to the inflammatory process, and has been associated with a higher risk of ulcerative colitis and colorectal cancer.[14,20,32] The preventive effect shown by AvPPE against the increased production of H_2S induced by HP was only observed at T1. At T2, due to its volatility, H_2S was probably eliminated during the culture period through the nitrogen flux used to generate the anaerobic condition in the bioreactor, and as a consequence, it could not be totally quantified. Our results confirm a previous study reporting that quebracho PACs reduced H_2S production when cultured with pig stools, these latter effects being associated with an inhibition of the bacterial metabolic activity and with a decrease in SRB and total bacteria.[33] Similar observation was also reported with human fecal microbiota cultured with a PAC-rich grape seed extract.[34] On the other hand, some dietary polyphenols have been shown to remove volatile sulfur compounds in the mouth;[35] accordingly, it is possible that this phenomenon also occurs in the colon with the avocado PACs, and this may explain the protective effect shown by the AvPPE on H_2S production.

Indole concentrations were also higher at T1 than T2, probably due to the volatility of this metabolite. This compound was recently described to exert beneficial activities, to act like a signaling molecule between the IM and its host, to be able to increase the expression of tight junction proteins, to improve gut barrier function, and to attenuate the inflammatory processes.[36] Additionally, indole may also influence host metabolism by modulating the secretion of GLP-1 by enteroendocrine L-cells.[37] Thus, the increase in indole concentration observed with HP/AvPPE at T1 may be considered as beneficial for the host. This increase could be due to a synergistic effect of higher tryptophan availability and the presence of polyphenols in the HP/AvPPE treatment,

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which could increase the indole-producing bacterial populations and/or their metabolic activity. In fact, AvPPE and HP treatments, by themselves, tend to increase the concentrations of indole in a nonsignificant manner when compared with C.

Regarding the SCFAs, these compounds are metabolic end products of dietary fiber and, in less proportion, proteins. The branched-chain SCFAs, isobutyrate and isovalerate, are originated exclusively from amino acids and can be considered as a marker of protein intake.[14] The amount and type of SCFA produced depend on the availability and type of substrates, the composition of the IM, and the duration of intestinal transit.[14] As expected, we observed increased concentrations of total SCFA, uSCFA and bSCFA, with HP, as a result of microbial fermentation and, more particularly, an increase of isobutyrate and isovalerate. AvPPE partially prevented the increased SCFA production induced by HP, suggesting that these compounds interfere with bacterial protein metabolism. These latter results are in accordance with the findings reported by Bazzocco et al. using high-molecular-weight PACs from apple.[38] Interestingly, the increase in propionate, valerate, isobutyrate, and isovalerate concentrations induced by HP confirms the results of Andriamihaja et al., in rats fed an HP diet (53% proteins) for 2 weeks.[39] On the other hand, it is noteworthy that AvPPE increases the levels of butyrate, an interesting result considering the well-known health-promoting effects of this SCFA.[15]

In general, all of the protein-derived metabolites correlated positively between them except for butyrate. This phenomenon can be explained by the fact that butyrate is the only metabolite that increases it concentration in the presence of AvPPE, while the other metabolites increased with proteins.

We also studied the effect of the different treatments on the composition of the fecal microbiota. Firmicutes and Bacteroidetes were the dominant phyla, as previously described.[40] Interestingly, our results showed that, in the presence of AvPPE, the abundance of the genus *Faecalibacterium* increased. This genus includes the species *Faecalibacterium prausnitzii*, a butyrate-producing microorganism that exerts anti-inflammatory activities and whose prevalence is decreased in patients with inflammatory bowel disease.[41]

Regarding the correlations, it is relevant to highlight the positive correlation between H_2S and *Bacteroides* genus. It has been described that bacteria of *Bacteroides* genus are capable to synthetize sulfur volatile compounds like H_2S in the oral cavity,[42] and our result suggests that this phenomenon could also occur in colon. On the other hand, H_2S was not correlated with *Desulfovibrio*, the most representative genus of SRB in human colon,[18] suggesting that SRB are not the main H_2S producer during the in vitro fermentation.[32]

In the second step, we evaluated whether the exposure of colonic epithelial cells to the CSs affected their viability, mitochondrial function, barrier function and DNA integrity. Our results show that the viability of HT-29Glc$^{-/+}$ cells was affected by the CS from HP, probably due to the presence of protein bacterial metabolites. Accordingly, recently, Wong et al., 2016, reported that HT-29Glc$^{-/+}$ exposure to *p*-cresol, one of these metabolites, induced cell damage and LDH release,[8] this deleterious effect being prevented by PAC-rich extracts from fruits, including AvPPE. However, in the present study, we did not observe any protective effect in the AvPPE-treated group, maybe because the CSs contain several other metabolites that could act synergistically, limiting the protective effect of AvPPE. Additionally, if we consider that we used a greater concentration of AvPPE in the culture medium of the cells (10-fold more than the minimum concentration used by Wong et al.), it is therefore possible that such concentration induced a certain degree of toxicity that could contribute to explain the lack of protective effect. Regarding the mitochondrial function, we observed that cell exposure to CSs from HP increased proton leak and decreased ATP-linked OCR. This indicates that the energy efficiency in treated cells is lower and that cell oxygen consumption is less associated with ATP synthesis. This phenomenon could contribute to the lower cell viability previously reported. At the concentration assayed, AvPPE had no effect on these altered parameters, even it seems somewhat toxic, thus indicating that these compounds do not protect the cells against viability loss. With respect to the intestinal barrier function, Caco-2 cell monolayers exhibited a greater permeability when exposed to CSs from HP, as reflected by decreased TEER values. This is an important finding considering that alterations in the gut barrier function generally precede the development of inflammatory events at local and systemic levels.[8] Interestingly, CS from AvPPE treatment reinforced barrier function (i.e., increased TEER values) compared with CS from C, and in addition, it prevented the barrier alterations induced by HP exposure. Similar results have been reported with dietary polyphenols including PACs in other models of intestinal barrier disturbances induced by nonsteroidal anti-inflammatory drugs, bile salts, oxidants, or *p*-cresol.[43,44] Finally, we also determined the impact of CS exposure on cell genotoxicity. Our results showed that CSs from C/AvPPE and HP/AvPPE treatments attenuated genotoxic damage, suggesting a potential antigenotoxic role of AvPPE and confirming results from a previous study.[45] CS from HP did not induce genotoxicity in our model, confirming the study from Windey et al. carried out through the Comet assay in HT-29 cells exposed to fecal water from subjects fed a high-protein diet.[46] Similar results were reported by Beaumont et al., where no DNA damage was observed in isolated colonocytes of rats fed for 2 weeks with HP diets, assessed by the Comet assay.[47]

In conclusion, our study confirms the deleterious effect of HP diets on the colonic epithelium, with the increase of protein-derived bacterial metabolites potentially toxic (like ammonia and H_2S) and their negative effect on cell viability, mitochondrial function, and intestinal barrier function of intestinal epithelial cells. We also demonstrated that the AvPPE exerted a protective effect in reducing the production of ammonia and H_2S and increasing the production of butyrate and indole, two beneficial metabolites for the colonic epithelia and at systemic level. The AvPPE also prevented the alterations in the intestinal permeability induced by HP condition and increased the relative abundance of the butyrate-producing genus *Faecalibacterium*. Finally, despite the suggested toxic effect of AvPPE to cell viability and mitochondrial function, the results of this work reveal a promising compound with a healthy potential to prevent the damage induced by protein metabolites and eventually HP diets at the colonic level. To that, further investigation is required, like adjusting the concentrations of the extract to avoid toxic effects, both in in vitro studies and subsequently animals and human studies.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b03905.

Concentration of unbranched (A) and branched short-chain fatty acids (B) in the CSs after 1 h (T1) and 24 h (T2) of fermentation (PDF)

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Notes

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■ ABBREVIATIONS

HPDs, high-protein diets; IM, intestinal microbiota; SCFA, short-chain fatty acids; uSCFAs, unbranched short-chain fatty acids; bSCFAs, branched short-chain fatty acids; H_2S, hydrogen sulfide; PACs, proanthocyanidins; AvPPE, avocado peel polyphenol extract; SRB, sulfate-reducing bacteria; C, control; HP, hyperproteic or high-protein; CS, culture supernatant; CSs, culture supernatants; NH_3, ammonia; DMEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase; OCR, oxygen consumption rate; TEER, transepithelial electrical resistance; OTUs, operational taxonomic units; SEM, standard error of the mean

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