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, H₂S, 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 H₂S, 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 (NH₃), hydrogen sulfide (H₂S), 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 H₂S by coupling oxidative phosphorylation with sulfate reduction¹⁷ and that H₂S 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′-TGCGAGAT-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), K₂HPO₄ (40 mg/L), KH₂PO₄ (40 mg/L), NaHCO₃ (2 g/L), MgSO₄·7H₂O (10 mg/L), CaCl₂·2H₂O (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 N₂ 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 450g 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 N₂ 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

197 inoculation and immediately aliquoted and frozen at -80°C until
198 analysis.

199 **Determination of Protein Metabolites.** *Ammonia* (NH_3).
200 Thawed CSs were centrifuged two times successively (15 min,
201 3405g, 4°C), and the supernatants were filtered ($0.22\ \mu\text{m}$) and
202 deproteinized (10 kDa Microcon centrifugal filter devices) (Merck,
203 Santiago, Chile). The determination of NH_3 was carried out in 96-
204 well plates using a commercial assay kit (K-AMIA, Megazyme,
205 Illinois) based on the reaction of ammonia (as ammonium ions;
206 NH_4^+) with 2-oxoglutarate to form L-glutamic acid and NADP^+ in the
207 presence of glutamate dehydrogenase and nicotinamide adenine
208 dinucleotide phosphate (NADPH). The decrease in absorbance at
209 340 nm, due to NADPH oxidation, was registered as a reflection of
210 the ammonia concentration in the sample.

211 **Hydrogen Sulfide** (H_2S). H_2S determination was carried out by gas
212 chromatography–mass spectrometry (GC–MS) after sulfide alkyla-
213 tion, as previously described.^{20,21} Thawed CSs were vortexed and
214 centrifuged (14 000g, 5 min, 4°C); 100 μL of supernatant was placed
215 in a vial with 100 μL of 5 mM benzalkonium chloride (prepared in
216 deoxygenated MiliQ water saturated with sodium tetraborate), 100
217 μL of 20 mM pentafluorobenzylbromide (in toluene), and 100 μL of
218 ethyl acetate (containing 100 μM naphthalene as internal standard);
219 and shaken at 55°C for 4 h in a rotating shaker. Thereafter, 150 μL
220 of KH_2PO_4 (saturated in MiliQ water) was added, the mixture was
221 centrifuged (10 000g, 10 min, 4°C), and the organic phase was
222 removed. One microliter was injected in an Agilent 6890N gas
223 chromatograph equipped with an HP-SHS capillary column (using
224 helium as gas carrier) and a 5973N MS detector (Agilent
225 Technologies, California). Calibration curves were made with
226 standard Na_2S solutions (as donor of H_2S) between 12.5 and 500
227 μM . The temperature of the injector and the transfer line was
228 maintained at 250°C and those of the ion source and the quadrupole
229 detector were 230 and 150°C , respectively. The GC program was as
230 follows: 70°C for 1 min, 100 – 180°C , ramp $8^{\circ}\text{C}/\text{min}$, 180 – 300°C ,
231 ramp $50^{\circ}\text{C}/\text{min}$, maintained for 2 min. The areas under the curves of
232 the samples and standard solutions ((bis-(pentafluorobenzyl) sulfide
233 (a derivative of sulfide)) were corrected by the area under the curve of
234 the internal standard, and the results were expressed as μM .

235 **Indole.** Thawed CSs were vortexed and centrifuged (10 min,
236 15 805g, room temperature). The supernatant was diluted four times
237 with 70% ethanol, and 100 μL was used for indole determination as
238 previously described.²² A calibration curve was made with indole
239 standards prepared in 70% ethanol, in a range of concentration
240 between 0 and 300 μM . For spectrophotometric determination, 100
241 μL of sample or standards were mixed in a 96-well plate with 25 μL of
242 5.3 M NaOH and 50 μL of 0.3 M hydroxylamine hydrochloride
243 ($\text{NH}_2\text{OH}\cdot\text{HCl}$). After 15 min of incubation, 125 μL of 2.7 M H_2SO_4
244 was added and the mixture was vortexed and incubated for 30 min at
245 room temperature. Absorbance was measured at 530 nm in an Infinite
246 200 PRO plate reader (TECAN, Männedorf, Switzerland).

247 **Short-Chain Fatty Acids (SCFAs).** SCFA determination was carried
248 out as previously described²³ with some modifications. The pH of the
249 thawed CSs was adjusted to 2–3 with 0.68 M HCl . After incubation
250 for 10 min at room temperature with occasional agitation, the samples
251 were centrifuged (10 min, 15 805g) and 2-ethyl butyric acid, as
252 internal standard, was added at a final concentration of 1 mM. The
253 detection and quantification of SCFAs were performed in an Agilent
254 7890A gas chromatograph (Agilent Technologies, California)
255 equipped with an FID detector and a Restek Stabilwax-Da (fused
256 silica) capillary column (Restek, Pensilvania) (30 m length, 0.32 mm
257 i.d.). The separation of the SCFAs was carried out between 120 and
258 240°C , with an initial heating of 120°C for 1 min, followed by an
259 increase to 240°C at a rate of $10^{\circ}\text{C}/\text{min}$, and then held for 3 min at
260 240°C . Hydrogen was used as a carrier gas, and the temperature of
261 the injector and detector was 240 and 265°C , respectively. A mix of
262 acetic acid, propionic acid, butyric acid, valeric acid, isobutyric acid,
263 and isovaleric acid (1 mg/mL) (Restek, Pensilvania) was used for
264 calibration. The area under the curve of the samples and standard
265 solutions was corrected by that of the internal standard.

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

284 The raw data paired-end reads obtained from the MiSeq platform
285 were stored in the ENA online public database under accession
286 number PRJEB30984 (<http://www.ebi.ac.uk/ena/data/view/PRJEB30984>).
287
288

Effect of CSs on Cell Viability, Mitochondrial Function, Barrier Function, and Genotoxic Damage in Intestinal Cell Lines.

Cell Viability. Thawed CSs (T_2 , 24 h) were centrifuged (15
291 min, 3405g, 4°C), and the supernatants of this centrifugation were
292 centrifuged again (15 min, 3405g, 4°C) and then subsequently
293 filtered ($0.22\ \mu\text{m}$). The effect of CS incubation on cell viability was
294 assayed in HT-29Glc^{-/+} cells, as previously described.⁸ Cells were
295 cultured in Dulbecco's modified Eagle's medium (DMEM)
296 supplemented with 4.5 g/L glucose, L-glutamine, and sodium pyruvate
297 (Corning, New York), 10% heat-inactivated fetal calf serum (Gibco,
298 ThermoFisher Scientific, Massachusetts), and 1% penicillin/strepto-
299 mycin (Hyclone, ThermoFisher Scientific, Massachusetts). At day 0,
300 cells were seeded in 96-well plates at a density of 2×10^4 cells/ cm^2 .
301 At day 3, the medium was changed, and from day 4 to 6, cells were
302 treated with the CSs at a final concentration of 10%, with daily change
303 of the culture medium. At day 7, cell viability was measured through
304 the quantification of the lactate dehydrogenase (LDH) (CytoTox
305 One Kit, Promega, Wisconsin) released in the culture medium. LDH
306 is a cytosolic enzyme that can be released to the culture medium when
307 there is a loss in the cellular membrane integrity, as reflex of loss in
308 cell viability. Thus, LDH quantification in the culture medium is an
309 indirect way to measure cell viability.²⁷ The results were normalized
310 expressing the value of LDH released in the culture medium as a
311 function of total LDH of the cells. To that, cells that remained in the
312 plate after the quantification of LDH release were lysed with Triton
313 0.02% to quantify the total LDH present in cells.

Mitochondrial Function. The effect of CSs on HT-29Glc^{-/+}
315 energy metabolism was evaluated by measuring the mitochondrial
316 function by polarography using an Oxygraph Strathkelvin 782 2-
317 channel oxygen system (Strathkelvin Instruments, North Lanarkshire,
318 Scotland) equipped with a 1302-type Clark electrode (Strathkelvin
319 Instruments, North Lanarkshire, Scotland) with a polypropylene
320 membrane, as previously described^{28,29} with some modifications. HT-
321 29Glc^{-/+} cells were cultured as previously described (**Cell Viability**
322 section). At day 0, cells were seeded in 24-well plates at a density of 2
323 $\times 10^4$ cells/ cm^2 . At day 3, the medium was changed, and from day 4
324 to 6, cells were treated with the CSs at a final concentration of 10%,
325 with daily change of the culture medium. At day 7, cells were
326 trypsinized and put into the oxygraph chamber at a density of 750 000
327 cells/mL in respiration medium (20 mM HEPES, 200 mM mannitol,
328 5 mM KH_2PO_4 , 2.5 mM MgCl_2 , 0.5 mM EGTA, 25 mM glucose, 2
329 mM L-glutamine, pH 7.4, enriched with bovine serum albumin 0.1%),
330 without cell culture medium and treatments. The oxygen
331 consumption rate (OCR, nmol $\text{O}_2/\text{min}/10^6$ cells) was evaluated at
332 baseline, after the addition of 2 μM oligomycin (an inhibitor of
333 adenosine 5'-triphosphate (ATP) synthase) to quantify the oxygen
334 consumption due to the proton leak (i.e., not coupled to ATP
335

Figure 1. Concentrations of NH₃ (A), H₂S (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 $\times$ 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/cm² in Transwell polycarbonate filters (12 mm diameter, 0.4 μm pore size) (Corning-Costar, New York) previously treated with 0.044 $\mu\text{g}/\mu\text{L}$ 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 cm² (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 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 $\pm$ standard deviation: 31.6 ± 4.5 years), had a normal weight (body mass index: 23.3 ± 2 kg/m²), and were healthy and omnivores.

Determination of Protein Metabolites in Culture Supernatants (CSs). No differences in NH₃ concentration between treatments were observed at 1 h of fermentation (T1) (Figure 1A). At 24 h (T2), NH₃ concentration significantly

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 $\times$ 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

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, 486

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 $\times$ 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).

487 *Faecalibacterium* and *Lachnospira*, showed significant differ-
 488 ences between the treatments. *Faecalibacterium* increased
 489 significantly in C/AvPPE and HP/AvPPE, compared with C
 490 (Figure 3D), while the relative abundance of *Lachnospira* was
 491 lower with HP than with HP/AvPPE (Figure 3E).

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

499 A basal OCR of 4.7 nmol O₂/min/10⁶ cells was observed in
 500 C, this value being unaffected by the different treatments
 501 (Figure 4B). Proton leak, evaluated in the presence of
 502 oligomycin, represented 26% of the basal OCR with C.
 503 AvPPE alone seems to augment somewhat the proton leak (in
 504 a not significant way). Proton leak augmented significantly
 505 with HP. Such increase was not prevented with the presence of
 506 AvPPE (Figure 4C). Consequently, the ATP-linked OCR
 507 significantly decreased with HP and HP/AvPPE, compared
 508 with C (Figure 4D). Maximal OCR and cell reserve capacity
 509 were not affected with any treatments (data not shown).

510 Changes in the intestinal barrier function were evaluated by
 511 measuring TEER in Caco-2 cells grown in Transwell filters and
 512 exposed to CSs (Figure 4E). TEER was similar in the four
 513 treatments at the beginning of the incubation period (0 h) and
 514 increased significantly with all of the treatments after 24 h of

515 exposition. At 24 h, TEER was significantly higher with C/
 516 AvPPE and lower with HP, compared with C. The decreased
 517 of TEER observed with HP was prevented with HP/AvPPE.

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

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

530 In relation to bacterial genera, a positive correlation between
 531 hydrogen sulfide and *Bacteroides* was observed; acetate and
 532 *Bilophila*; propionate and *Coprococcus*; butyrate and *Sutterella*,
 533 *Prevotella*, *Desulfovibrio*, *Catenibacterium*, and *Bifidobacterium*;
 534 valerate and *Ruminococcus*; isobutyrate and *Sutterella*; and
 535 between isovalerate and *Sutterella* and *Butyririmonas*. On the
 536 other hand, a negative correlation was observed between
 537 hydrogen sulfide and *Faecalibacterium*, *Anaerostipes* and
 538 [*Prevotella*]; indole and *Lachnospira*; acetate and *Prevotella*
 539 and [*Ruminococcus*]; propionate and *Dorea* and [*Ruminococ-*
 540 *cus*]; valerate and *Bifidobacterium* and *Bacteroides*; and between
 541 isobutyrate and *Dorea* (Figure 5B).

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, 603

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 its 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 H₂S and *Bacteroides* genus. It has been described that bacteria of *Bacteroides* genus are capable to synthesize sulfur volatile compounds like H₂S in the oral cavity,⁴² and our result suggests that this phenomenon could also occur in colon. On the other hand, H₂S was not correlated with *Desulfovibrio*, the most representative genus of SRB in human colon,¹⁸ suggesting that SRB are not the main H₂S 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 H₂S) 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 H₂S 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.

■ 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; H₂S, 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; NH₃, 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

■ REFERENCES

- (1) Yahia, E. M.; Maldonado Celis, M. E.; Svendsen, M. The Contribution of Fruit and Vegetable Consumption to Human Health. *Fruit Veg. Phytochem.* **2017**, 1–52.
- (2) Tremocoldi, M. A.; Rosalen, P. L.; Franchin, M.; Massarioli, A. P.; Denny, C.; Daiuto, E. R.; Paschoal, J. A. R.; Melo, P. S.; de Alencar, S. M. Exploration of avocado by-products as natural sources of bioactive compounds. *PLoS One* **2018**, 13, No. e0192577.
- (3) Figueroa, J. G.; Borrás-Linares, I.; Lozano-Sánchez, J.; Segura-Carretero, A. Comprehensive identification of bioactive compounds of avocado peel by liquid chromatography coupled to ultra-high-definition accurate-mass Q-TOF. *Food Chem.* **2018**, 245, 707–716.
- (4) Figueroa, J. G.; Borrás-Linares, I.; Lozano-Sánchez, J.; Segura-Carretero, A. Comprehensive characterization of phenolic and other polar compounds in the seed and seed coat of avocado by HPLC-DAD-ESI-QTOF-MS. *Food Res. Int.* **2018**, 105, 752–763.
- (5) Rodrigo, R.; Libuy, M.; Feliu, F.; Hasson, D. Polyphenols in disease: from diet to supplements. *Curr. Pharm. Biotechnol.* **2014**, 15, 304–317.

- (6) Cires, M. J.; Wong, X.; Carrasco-Pozo, C.; Gotteland, M. The gastrointestinal tract as a key target organ for the health-promoting effects of dietary proanthocyanidins. *Front. Nutr.* **2016**, 3, 57. DOI: 10.3389/fnut.2016.00057.
- (7) Neilson, A. P.; O'Keefe, S. F.; Bolling, B. W. High-Molecular-Weight Proanthocyanidins in Foods: Overcoming Analytical Challenges in Pursuit of Novel Dietary Bioactive Components. *Annu. Rev. Food Sci. Technol.* **2016**, 7, 43–64.
- (8) Wong, X.; Carrasco-Pozo, C.; Escobar, E.; Navarrete, P.; Blachier, F.; Andriamihaja, M.; Lan, A.; Tomé, D.; Cires, M. J.; Pastene, E.; Gotteland, M. Deleterious effect of *p*-cresol on human colonic epithelial cells prevented by proanthocyanidin-containing polyphenol extracts from fruits and proanthocyanidin bacterial metabolites. *J. Agric. Food Chem.* **2016**, 64, 3574–3583.
- (9) Andriamihaja, M.; Lan, A.; Beaumont, M.; Grauso, M.; Gotteland, M.; Pastene, E.; Cires, M. J.; Carrasco-Pozo, C.; Tomé, D.; Blachier, F. Proanthocyanidin-containing polyphenol extracts from fruits prevent the inhibitory effect of hydrogen sulfide on human colonocyte oxygen consumption. *Amino Acids* **2018**, 50, 755–763.
- (10) Clifton, P. M.; Keogh, J. Metabolic effects of high-protein diets. *Curr. Atheroscler. Rep.* **2007**, 9, 472–478.
- (11) Westerterp-Plantenga, M. S.; Lemmens, S. G.; Westerterp, K. R. Dietary protein - its role in satiety, energetics, weight loss and health. *Br. J. Nutr.* **2012**, 108, S105–12.
- (12) Windey, K.; De Preter, V.; Verbeke, K. Relevance of protein fermentation to gut health. *Mol. Nutr. Food Res.* **2012**, 56, 184–196.
- (13) Yao, C. K.; Muir, J. G.; Gibson, P. R. Review article: insights into colonic protein fermentation, its modulation and potential health implications. *Aliment. Pharmacol. Ther.* **2016**, 43, 181–196.
- (14) Davila, A. M.; Blachier, F.; Gotteland, M.; Andriamihaja, M.; Benetti, P. H.; Sanz, Y.; Tomé, D. Intestinal luminal nitrogen metabolism: role of the gut microbiota and consequences for the host. *Pharmacol. Res.* **2013**, 68, 95–107.
- (15) Hamer, H. M.; Jonkers, D.; Venema, K.; Vanhoutvin, S.; Troost, F. J.; Brummer, R. J. Review article: the role of butyrate on colonic function. *Aliment. Pharmacol. Ther.* **2008**, 27, 104–119.
- (16) Torres, E.; García, A.; Aranda, M.; Saéz, V.; Zúñiga, F.; Alarcón, J.; Avello, M.; Pastene, E. One-step purification of two semi-synthetic epicatechin adducts prepared from avocado peels procyanidins by centrifugal partition chromatography and evaluation of their anti-inflammatory effects on adenocarcinoma gastric cells infected with *Helicobacter pylori*. *J. Chil. Chem. Soc.* **2018**, 63, 4222–4228.
- (17) Florin, T.; Neale, G.; Gibson, G. R.; Christl, S. U.; Cummings, J. H. Metabolism of dietary sulphate: absorption and excretion in humans. *Gut* **1991**, 32, 766–773.
- (18) Rey, F. E.; Gonzalez, M. D.; Cheng, J.; Wu, M.; Ahern, P. P.; Gordon, J. I. Metabolic niche of a prominent sulfate-reducing human gut bacterium. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, 110, 13582–13587.
- (19) Tzounis, X.; Vulevic, J.; Kuhnle, G. G.; George, T.; Leonczak, J.; Gibson, G. R.; Kwik-Uribe, C.; Spencer, J. P. Flavanol monomer-induced changes to the human faecal microflora. *Br. J. Nutr.* **2008**, 99, 782–792.
- (20) Beaumont, M.; Andriamihaja, M.; Lan, A.; Khodorova, N.; Audebert, M.; Blouin, J. M.; Grauso, M.; Lancha, L.; Benetti, P. H.; Benamouzig, R.; Tome, D.; Bouillaud, F.; Davila, A. M.; Blachier, F. Detrimental effects for colonocytes of an increased exposure to luminal hydrogen sulfide: The adaptive response. *Free Radicals Biol. Med.* **2016**, 93, 155–164.
- (21) Hyspler, R.; Ticha, A.; Indrova, M.; Zadak, Z.; Hysplerova, L.; Gasparic, J.; Churacek, J. A simple, optimized method for the determination of sulphide in whole blood by GC-MS as a marker of bowel fermentation processes. *J. Chromatogr. B* **2002**, 770, 255–259.
- (22) Darkoh, C.; Chappell, C.; Gonzales, C.; Okhuysen, P. A Rapid and Specific Method for the Detection of Indole in Complex Biological Samples. *Appl. Environ. Microbiol.* **2015**, 81, 8093–8097.
- (23) Zhao, G.; Nyman, M.; Jonsson, J. A. Rapid determination of short-chain fatty acids in colonic contents and faeces of humans and rats by acidified water-extraction and direct-injection gas chromatography. *Biomed. Chromatogr.* **2006**, 20, 674–682.

- (24) Fujio-vejar, S.; Vasquez, Y.; Morales, P.; Magne, F.; Vera-Wolf, P.; Ugalde, J. A.; Navarrete, P.; Gotteland, M. The Gut Microbiota of Healthy Chilean Subjects Reveals a High Abundance of the Phylum Verrucomicrobia. *Front. Microbiol.* **2017**, *8*, No. 1221.
- (25) Caporaso, J. G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F. D.; Costello, E. K.; Fierer, N.; Pena, A. G.; Goodrich, J. K.; Gordon, J. I.; Huttley, G. A.; Kelley, S. T.; Knights, D.; Koenig, J. E.; Ley, R. E.; Lozupone, C. A.; McDonald, D.; Muegge, B. D.; Pirrung, M.; Reeder, J.; Sevinsky, J. R.; Tumbaugh, P. J.; Walters, W. A.; Widmann, J.; Yatsunenko, T.; Zaneveld, J.; Knight, R. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7*, 335–336.
- (26) McDonald, D.; Price, M. N.; Goodrich, J.; Nawrocki, E. P.; DeSantis, T. Z.; Probst, A.; Andersen, G. L.; Knight, R.; Hugenholtz, P. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* **2012**, *6*, 610–618.
- (27) Legrand, C.; Bour, J. M.; Jacob, C.; Capiamont, J.; Martial, A.; Marc, A.; Wudtke, M.; Kretzmer, G.; Demangel, C.; Duval, D.; Hache, J. Lactate dehydrogenase (LDH) activity of the number of dead cells in the medium of cultured eukaryotic cells as marker. *J. Biotechnol.* **1992**, *25*, 231–243.
- (28) Carrasco-Pozo, C.; Tan, K. N.; Reyes-Farias, M.; De La Jara, N.; Ngo, S. T.; Garcia-Diaz, D. F.; Llanos, P.; Cires, M. J.; Borges, K. The deleterious effect of cholesterol and protection by quercetin on mitochondrial bioenergetics of pancreatic beta-cells, glycemic control and inflammation: In vitro and in vivo studies. *Redox Biol.* **2016**, *9*, 229–243.
- (29) Andriamihaja, M.; Lan, A.; Beaumont, M.; Audebert, M.; Wong, X.; Yamada, K.; Yin, Y. L.; Tome, D.; Carrasco-Pozo, C.; Gotteland, M.; Kong, X. F.; Blachier, F. The deleterious metabolic and genotoxic effects of the bacterial metabolite *p*-cresol on colonic epithelial cells. *Free Radical Biol. Med.* **2015**, *85*, 219–227.
- (30) Hassanat, F.; Benchaar, C. Assessment of the effect of condensed (acacia and quebracho) and hydrolysable (chestnut and valonea) tannins on rumen fermentation and methane production in vitro. *J. Sci. Food Agric.* **2013**, *93*, 332–339.
- (31) Stewart, J. A.; Chadwick, V. S.; Murray, A. Carriage, quantification, and predominance of methanogens and sulfate-reducing bacteria in faecal samples. *Lett. Appl. Microbiol.* **2006**, *43*, 58–63.
- (32) Blachier, F.; Beaumont, M.; Kim, E. Cysteine-derived hydrogen sulfide and gut health: a matter of endogenous or bacterial origin. *Curr. Opin. Clin. Nutr. Metab. Care* **2019**, *22*, 68–75.
- (33) Whitehead, T. R.; Spence, C.; Cotta, M. A. Inhibition of hydrogen sulfide, methane, and total gas production and sulfate-reducing bacteria in in vitro swine manure by tannins, with focus on condensed quebracho tannins. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 8403–8409.
- (34) Yamakoshi, J.; Tokutake, S.; Kikuchi, M.; Kubota, Y.; Konishi, H.; Mitsuoka, T. Effect of Proanthocyanidin-Rich Extract from Grape Seeds on Human Fecal Flora and Fecal Odor. *Microb. Ecol. Health Dis.* **2001**, *13*, 25–31.
- (35) Zeng, Q. C.; Wu, A. Z.; Pika, J. The effect of green tea extract on the removal of sulfur-containing oral malodor volatiles in vitro and its potential application in chewing gum. *J. Breath Res.* **2010**, *4*, No. 036005.
- (36) Bansal, T.; Alaniz, R. C.; Wood, T. K.; Jayaraman, A. The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 228–233.
- (37) Chimere, C.; Emery, E.; Summers, D. K.; Keyser, U.; Gribble, F. M.; Reimann, F. Bacterial metabolite indole modulates incretin secretion from intestinal enteroendocrine L cells. *Cell Rep.* **2014**, *9*, 1202–1208.
- (38) Bazzocco, S.; Mattila, I.; Guyot, S.; Renard, C. M.; Aura, A. M. Factors affecting the conversion of apple polyphenols to phenolic acids and fruit matrix to short-chain fatty acids by human faecal microbiota in vitro. *Eur. J. Nutr.* **2008**, *47*, 442–452.
- (39) Andriamihaja, M.; Davila, A. M.; Eklou-Lawson, M.; Petit, N.; Delpal, S.; Allek, F.; Blais, A.; Delteil, C.; Tome, D.; Blachier, F. Colon luminal content and epithelial cell morphology are markedly modified in rats fed with a high-protein diet. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **2010**, *299*, G1030–G1037.
- (40) Eckburg, P. B.; Bik, E. M.; Bernstein, C. N.; Purdom, E.; Dethlefsen, L.; Sargent, M.; Gill, S. R.; Nelson, K. E.; Relman, D. A. Diversity of the human intestinal microbial flora. *Science* **2005**, *308*, 1635–1638.
- (41) Miquel, S.; Martín, R.; Rossi, O.; Bermúdez-Humarán, L. G.; Chatel, J. M.; Sokol, H.; Thomas, M.; Wells, J. M.; Langella, P. *Faecalibacterium prausnitzii* and human intestinal health. *Curr. Opin. Microbiol.* **2013**, *16*, 255–261.
- (42) Tonzetich, J.; McBride, B. C. Characterization of volatile sulphur production by pathogenic and non-pathogenic strains of oral *Bacteroides*. *Arch. Oral Biol.* **1981**, *26*, 963–969.
- (43) Carrasco-Pozo, C.; Morales, P.; Gotteland, M. Polyphenols protect the epithelial barrier function of Caco-2 cells exposed to indomethacin through the modulation of occludin and zonula occludens-1 expression. *J. Agric. Food Chem.* **2013**, *61*, 5291–5297.
- (44) Erlejman, A. G.; Fraga, C. G.; Oteiza, P. I. Procyanidins protect Caco-2 cells from bile acid- and oxidant-induced damage. *Free Radical Biol. Med.* **2006**, *41*, 1247–1256.
- (45) Bakheet, S. A.; Alhuraishi, A. M.; Al-Harbi, N. O.; Al-Hosaini, K. A.; Al-Sharary, S. D.; Attia, M. M.; Alhoshani, A. R.; Al-Shabanah, O. A.; Al-Harbi, M. M.; Imam, F.; Ahmad, S. F.; Attia, S. M. Alleviation of Aflatoxin B1-Induced Genomic Damage by Proanthocyanidins via Modulation of DNA Repair. *J. Biochem. Mol. Toxicol.* **2016**, *30*, 559–566.
- (46) Windey, K.; De Preter, V.; Louat, T.; Schuit, F.; Herman, J.; Vansant, G.; Verbeke, K. Modulation of Protein Fermentation Does Not Affect Fecal Water Toxicity: A Randomized Cross-Over Study in Healthy Subjects. *PLoS One* **2012**, *7*, No. e52387.
- (47) Beaumont, M.; Andriamihaja, M.; Armand, L.; Grauso, M.; Jaffrézic, F.; Laloë, D.; Moroldo, M.; Davila, A.-M.; Tomé, D.; Blachier, F.; Lan, A. Epithelial response to a high-protein diet in rat colon. *BMC Genomics* **2017**, *18*, No. 116.