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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, 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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64 bacterial metabolites (3-phenylpropionic acid, 3,4-dihydrox-
65 yphenyl propionic acid, and 4-hydroxyphenyl acetic acid)
66 prevented the deleterious effects of the microbiota-derived
67 protein metabolites, *p*-cresol and H₂S, on HT-29Glc^{-/+} and
68 Caco-2 cells, suggesting that dietary PACs might protect
69 against the negative impact of high-protein diets (HPDs) on
70 colonic epithelial cells.^{8,9}

71 High-protein diets (HPDs) bring 25–35% of their energy as
72 proteins, compared with normo-protein diets that only
73 contribute with 12–18% of the energy intake.¹⁰ They are
74 widely used by athletes for increasing their muscle mass and by
75 overweight and obese subjects for losing weight, through their
76 ability to stimulate satiety and increase thermogenesis.^{10,11}

77 About 90% of the dietary proteins are digested and absorbed in
78 the small intestine, and the remaining 10% reach the colon
79 undigested or partially digested. Accordingly, a higher intake of
80 dietary proteins generally means a higher flux of undigested
81 proteins reaching the colon.¹² In the colon, undigested
82 proteins are hydrolyzed by proteases from the IM. The
83 released amino acids are used for bacterial protein synthesis
84 and growth or are fermented, generating an array of bacterial
85 metabolites that accumulate in the colonic lumen.^{13,14} While
86 short-chain fatty acids (SCFA) are considered as beneficial
87 metabolites,^{14,15} other byproducts including ammonia (NH₃),
88 hydrogen sulfide (H₂S), phenol, *p*-cresol, and polyamines at
89 excessive concentrations are potentially deleterious for the
90 colonic epithelium and at the systemic level.^{12–14} In the colon,
91 proteins can also modulate the composition of the intestinal
92 microbiota, favoring the development of pathogenic and pro-
93 inflammatory populations like *Clostridium perfringens*, *Enter-*
94 *ococcus*, *Shigella*, and *Escherichia coli* spp. and diminishing those
95 beneficial like *Bifidobacterium* spp.¹³ The colonic fermentation
96 of proteins by the IM is, therefore, considered as a potentially
97 harmful process for host health, which could enhance the risk
98 of diseases such as colorectal cancer and ulcerative colitis.^{12–14}

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

106 ■ MATERIALS AND METHODS

107 **Avocado Peel Polyphenol Extract (AvPPE).** Avocado peels
108 were obtained from fresh avocado fruits (*P. americana* Mill. Var. Hass)
109 purchased from local market, and AvPPE was prepared by the
110 Laboratory of Pharmacognosy at the University of Concepción
111 (Concepción, Biobío, Chile) and subsequently freeze-dried and stored
112 at –70 °C until use. The elaboration of the extract and the
113 characterization of its polyphenol composition have been previously
114 described.⁸ Thirty seven grams of fresh avocado peel was necessary to
115 obtain 1 g of AvPPE.¹⁶ Briefly, frozen samples of avocado peels (500
116 g) were mixed with hot water (90 °C, 5 min) and then filtered
117 (sintered glass funnel, 70–100 μm). Marcs were homogenized and
118 extracted again with hot water (65 °C). After maceration (60 min),
119 sugars and water-soluble compounds were removed with distilled
120 water by the use of a glass column packed with Sepabeads SP-850
121 (Supelco, Bellefonte). Polyphenol extract was obtained by the same
122 procedure but eluted with absolute ethanol and then was evaporated
123 under vacuum, freeze-dried, and stored at –70 °C until use.⁸ The
124 identification and characterization of polyphenolic extract was carried
125 out by RP-HPLC in a Waters Alliance 2695 system equipped with a
126 C18 column and C18 pre-column. Quantification was carried out
127 using a UV–vis chromatogram. Standards were used to perform

calibration curves.⁸ AvPPE contained 14% of flavan-3-ol monomers, 128
29.1% of proanthocyanidins (PACs, type A and B, including only 129
epicatechin monomers, with a mean degree of polymerization of 6.1), 130
15.1% of other flavonoids, 3% of anthocyanins, and 38.8% of phenolic
acids, as shown in Table S1. 131 132

Donor Recruitment. This study was conducted in accordance 133
with the Declaration of Helsinki and approved by the Ethics 134
Committee for Research in Humans, Faculty of Medicine, University 135
of Chile (No. 118-2014). Healthy subjects recruited in the study must 136
provide a written informed consent. Smokers and subjects with intake 137
of antibiotics or probiotics in the last 2 months before stool collection 138
were excluded from the study. Subjects were selected according to the 139
presence of sulfate-reducing bacteria (SRB) in their microbiota, due 140
to the fact that these microorganisms produce H₂S by coupling 141
oxidative phosphorylation with sulfate reduction¹⁷ and that H₂S was 142
one of the protein bacterial metabolites determined in our study. 143
Subjects were considered as SRB-positive based on the polymerase 144
chain reaction (PCR) amplification of the *aprA* gene that encodes the 145
 α -subunit of the adenosine-5'-phosphosulfate reductase present in all 146
known SRB.¹⁸ Fecal bacterial DNA coming from a single stool sample 147
was extracted by the QIAamp Fast DNA Stool Mini Kit (QIAGEN, 148
Hilden, Germany) using the recommendations of the manufacturer, 149
with an additional step of mechanic lysis with a bead beater. PCR was 150
carried out using the *aprA* gene primers Fwd.: 5'-TGGCAGAT- 151
MATGATYMACGG-3' and Rev.: 5'-GGGCCGTAACCGTCCTT- 152
GAA-3'.¹⁸ DNA samples from *Desulfovibrio piger* and *Desulfovibrio* 153
piezophilus (DSMZ, Braunschweig, Germany) and from *C. perfringens* 154
and *E. coli* were used as positive and negative controls, respectively. 155
Quantification of SRB in PCR-positive subjects was performed by 156
qPCR using a LightCycler 2.0 (Roche, Basel, Switzerland) and the 157
LightCycler FastStart DNA Master SYBR Green I kit (Roche, Basel, 158
Switzerland); standard curves were carried out with *Dromaeolus piger* 159
DNA. 160

Fecal Sampling. Five days before and during fecal sampling, the 161
recruited subjects were counseled by a registered dietitian to consume 162
a standardized diet (1800 and 2000 kcal/day for women and men, 163
respectively; 15% proteins, 58% carbohydrates, and 27% fat), with the 164
aim of decreasing the inter- and intraindividual variability in their 165
microbiota composition. Stools were collected in sterile plastic 166
recipients, maintained in anaerobic conditions (GasPak, Becton- 167
Dickinson, New Jersey), and refrigerated (4 °C) until processing, 168
within the 3 h following their emission. Every 2 days, each recruited 169
subject provided one fresh stool sample, until four samples were 170
provided. Each sample was used for one independent assay. 171

In Vitro Fermentation of Human Fecal Microbiota. Fecal 172
inocula and bioreactor cultures were performed in a pre-reduced 173
culture medium containing peptone (2 g/L), yeast extract (2 g/L), 174
NaCl (100 mg/L), K₂HPO₄ (40 mg/L), KH₂PO₄ (40 mg/L), 175
NaHCO₃ (2 g/L), MgSO₄·7H₂O (10 mg/L), CaCl₂·2H₂O (10 mg/ 176
L), tween 80 (2 mL/L), hemin (50 mg/L), vitamin K1 (10 μL/L), 177
bile salts (500 mg/L), resazurin (1 mg/L), and glucose (10 g/L) in 178
distilled and sterile water as previously described,¹⁹ with minor 179
modifications. The sterile culture medium was gassed for 1.5 h with 180
N₂ before inoculation. For the inoculum preparation, fresh stools were 181
diluted in culture medium at a concentration of 100 g/L and then 182
were mixed in a stomacher for 30 s. After that, the mix was 183
centrifuged at 450g for 2 min. The supernatant of this centrifugation 184
was inoculated directly in the culture medium of the bioreactor at a 185
final concentration of 1% of stools. Depending on the treatment, the 186
culture medium was used alone (control (C)) or supplemented with 187
1.2 g/L AvPPE (AvPPE), 2 g/L proteins (hyperproteic (HP)), or 2 188
g/L proteins and 1.2 g/L AvPPE (HP/AvPPE). The protein was a 189
mix of casein (80%) (Sigma, Missouri) and whey protein (20%) 190
(Hilmar, California). Culture was conducted for 24 h at 37 °C in 191
anaerobic conditions under N₂ flow and constant agitation, using a 192
Micro DCU-200 bioreactor system (B. Braun Biotech International, 193
Berlin, Germany). pH was automatically maintained at 6.8 by 194
injecting HCl (0.5 M) or NaOH (1 M). Samples of culture 195
supernatants (CSs) were collected at 1 h (T1) and 24 h (T2) after 196

197 inoculation and immediately aliquoted and frozen at $-80\text{ }^{\circ}\text{C}$ until
198 analysis.

199 **Determination of Protein Metabolites.** *Ammonia* (NH_3).
200 Thawed CSs were centrifuged two times successively (15 min,
201 3405g, $4\text{ }^{\circ}\text{C}$), and the supernatants were filtered ($0.22\text{ }\mu\text{m}$) 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, $4\text{ }^{\circ}\text{C}$); 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 $55\text{ }^{\circ}\text{C}$ 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, $4\text{ }^{\circ}\text{C}$), 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 $250\text{ }^{\circ}\text{C}$ and those of the ion source and the quadrupole
229 detector were $230\text{ }^{\circ}\text{C}$ and $150\text{ }^{\circ}\text{C}$, respectively. The GC program was as
230 follows: $70\text{ }^{\circ}\text{C}$ for 1 min, $100\text{--}180\text{ }^{\circ}\text{C}$, ramp $8\text{ }^{\circ}\text{C}/\text{min}$, $180\text{--}300\text{ }^{\circ}\text{C}$,
231 ramp $50\text{ }^{\circ}\text{C}/\text{min}$, maintained for 2 min. The areas under the curves of
232 the samples and standard solutions ((bis-(pentafluorobenzyl) sulfide
233 (a derivate 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 $240\text{ }^{\circ}\text{C}$, with an initial heating of $120\text{ }^{\circ}\text{C}$ for 1 min, followed by an
259 increase to $240\text{ }^{\circ}\text{C}$ at a rate of $10\text{ }^{\circ}\text{C}/\text{min}$, and then held for 3 min at
260 $240\text{ }^{\circ}\text{C}$. Hydrogen was used as a carrier gas, and the temperature of
261 the injector and detector was 240 and $265\text{ }^{\circ}\text{C}$, 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 demultiplexed 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/](http://www.ebi.ac.uk/ena/data/view/PRJEB30984) 287
[PRJEB30984](http://www.ebi.ac.uk/ena/data/view/PRJEB30984)). 288

Effect of CSs on Cell Viability, Mitochondrial Function, 289
Barrier Function, and Genotoxic Damage in Intestinal Cell 290
Lines. *Cell Viability.* Thawed CSs (T2, 24 h) were centrifuged (15 291
min, 3405g, $4\text{ }^{\circ}\text{C}$), and the supernatants of this centrifugation were 292
centrifuged again (15 min, 3405g, $4\text{ }^{\circ}\text{C}$) and then subsequently 293
filtered ($0.22\text{ }\mu\text{m}$). 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/cm². At 301
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. 314

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/cm². 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

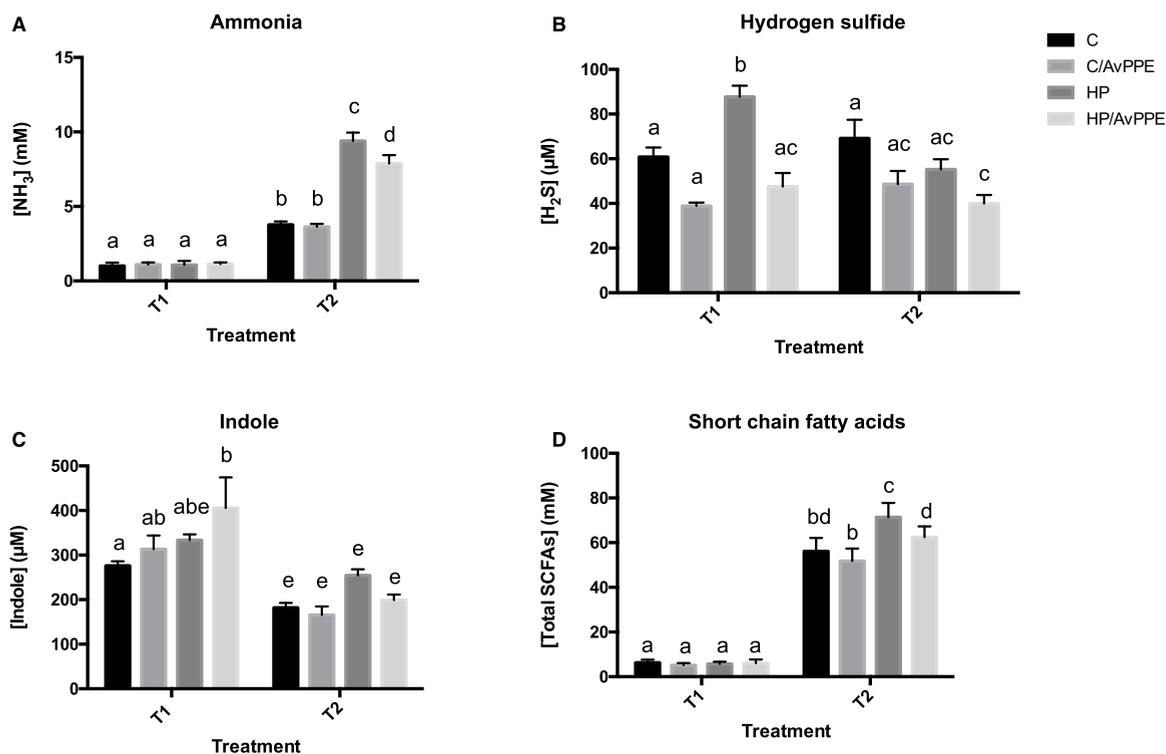


Figure 1. Concentrations of NH₃ (A), H₂S (B), indole (C), and total SCFAs (D) in the CSs after 1 (T1) and 24 h (T2) of fermentation. Each bar represents the average of five subjects (with SEM). Data were analyzed by two-way ANOVA with repeated measures (time effect: $p = 0.0002$ (A), $p = 0.21$ (B), $p = 0.0019$ (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 ($p < 0.05$).

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

344 **Intestinal Barrier Function.** The effect of CSs on the intestinal
345 barrier function was determined in Caco-2 cells (ATCC, Virginia) by
346 measuring changes in transepithelial electrical resistance (TEER).
347 Cells (passages 10–21) were cultured in DMEM/Ham's F12 medium
348 supplemented with L-glutamine (Corning, New York), 10% heat-
349 inactivated fetal calf serum (Gibco, Thermo Fisher Scientific,
350 Massachusetts), 1% penicillin/streptomycin (Hyclone, ThermoFisher
351 Scientific, Massachusetts), and 1% nonessential amino acid solution
352 (Corning, New York), as previously described.⁸ At day 0, cells were
353 seeded at a density of 10^5 cells/cm² in Transwell polycarbonate filters
354 (12 mm diameter, 0.4 μm pore size) (Corning-Costar, New York)
355 previously treated with 0.044 $\mu\text{g}/\mu\text{L}$ collagen in 0.01 M HCl. The
356 apical and basolateral compartments of the filters were filled with 500
357 and 1000 μL of culture medium, respectively, and the culture medium
358 was changed three times per week. Once the TEER reached 1000 Ω
359 cm² (approximately 6–7 days after seeding), 30% of the apical culture
360 medium was replaced with the CSs. TEER was measured with a
361 milliohm meter (EVOM, World Precision Instruments, Florida) at
362 baseline and 24 h after medium replacement.

363 **Genotoxic Damage.** The genotoxic damage induced by the CSs in
364 HT-29Glc^{-/+} cells was assessed through the quantification of
365 phosphorylated histones using the H2A.X phosphorylation assay kit
366 (Merck, Santiago, Chile). H2A.X histones phosphorylate in response
367 to the breakdown of the double strand of DNA. HT-29Glc^{-/+} cells
368 were cultured as mentioned above (Cell Viability section) at a density
369 of 36×10^3 cells/well in 96-well plates as previously described.²⁹ After
370 16 h, complete culture medium was replaced with culture medium

371 without fetal calf serum and with 15% CS, and after 24 h, the levels of
372 genotoxic damage were evaluated by quantifying phosphorylated
373 H2A.X histones by luminescence. Results were expressed as the % of
374 the positive control (etoposide 10 μM).

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

RESULTS

388
389 **Donor Recruitment.** Eight out of twelve subjects
390 evaluated (66.7%) were positive for the presence of SRB in
391 their stools, assessed by PCR. After a qPCR quantification of
392 SRB, we selected the subject participants based on their
393 similitude in their counts, which ranged between 1.05×10^6
394 and 5.82×10^8 copies of *aprA* gene/g stool. We selected five
395 subjects who had an average of 7.02×10^7 copies of *aprA*
396 gene/g stool. They were aged 26–37 years (mean \pm standard
397 deviation: 31.6 ± 4.5 years), had a normal weight (body mass
398 index: 23.3 ± 2 kg/m²), and were healthy and omnivores.

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

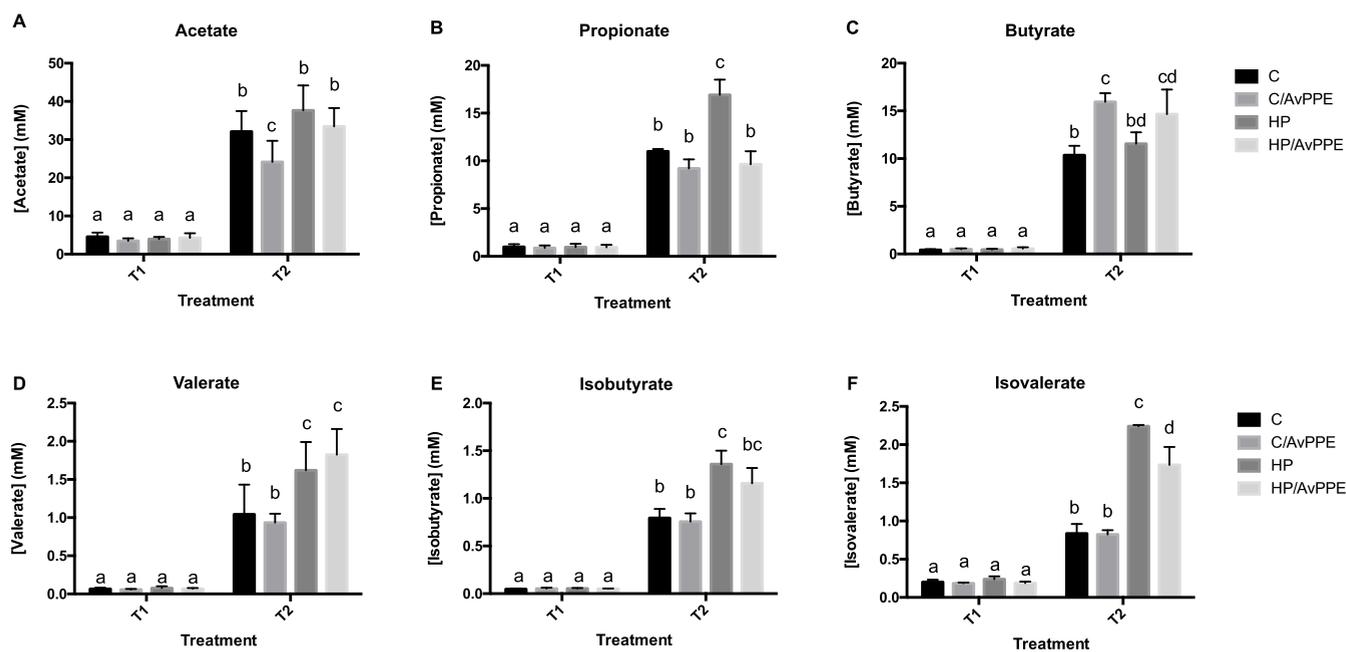


Figure 2. Concentration of uSCFAs (acetate (A), propionate (B), butyrate (C), and valerate (D)) and bSCFAs (isobutyrate (E) and isovalerate (F)) in the CSs at 1 (T1) and 24 h (T2) of fermentation. Each bar represents the average of five subjects (with SEM). Data were analyzed by two-way ANOVA with repeated 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 ($p < 0.05$).

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

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

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

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

427 When evaluating each SCFA individually (Figure 2), acetate,
428 propionate, and butyrate were the most abundant. All SCFAs
429 increased significantly at T2 compared with T1 and with all of
430 the treatments. At T2, acetate significantly decreased and
431 butyrate increased in C/AvPPE compared with C (Figure
432 2A,C). HP induced a significant increase in the production of
433 propionate, valerate, and the bSCFAs isobutyrate and
434 isovalerate (Figure 2B,D–F). The increase of propionate and
435 isovalerate was prevented with HP/AvPPE (Figure 2B,F).

436 **Effect of the Treatments on the Microbiota Compo-**
437 **sition.** A total of 1 874 289 sequences were obtained after

trimming, assembly, quality filtering, and chimera checking, 438
which exhibited an average of 455 ± 9.8 bp sequence length. 439
After OTU assignment, a total of 1 504 917 counts were 440
obtained in BIOM table, ranging from 18 853 to 94 706 counts 441
per sample. The rarefaction curves reached an asymptote 442
(Figure S2A), indicating that the number of sequences 443
obtained per sample was sufficient to describe the diversity 444
of the bacterial communities of the samples, without significant 445
differences between them. Considering the taxa with a relative 446
abundance $>0.1\%$, 8 phyla, 22 families, and 32 genera were 447
identified. No differences between the four treatments were 448
detected in phylum, family, or genus at T1. The core 449
microbiota, which represents the genera present in all of the 450
subjects in the control condition at T1, represented the 29.8% 451
of the total microorganisms (Table S2). The intraindividual α 452
diversity, evaluated through the Chao1 index, did not show any 453
significant differences between the four treatments at T2 ($p =$ 454
0.52). The inter-individual β diversity, evaluated at T2 through 455
weighted Unifrac, did not show significant differences (Figure 456
S2B). The prevalence and relative abundance of the bacterial 457
phyla, families, and genera at T2 for each of the four 458
treatments are shown in Tables S3–S5, respectively. 459

460 Considering the average of the four treatments at T2, the 460
phyla Firmicutes (42%), Bacteroidetes (34.8%), Proteobacteria 461
(22.2%), and Actinobacteria (0.44%) were the most abundant, 462
while the others, globally, had a relative abundance of 0.15%. 463
The relative abundance of the Actinobacteria phylum at T2 464
significantly differed between the treatments, increasing with 465
HP/AvPPE compared with C (Figure 3A). At the family level, 466
considering the average of the four treatments at T2, 467
Bacteroidaceae (21.7%) was the most abundant, followed by 468
Lachnospiraceae (12.3%), Enterobacteriaceae (11.2%), Rumi- 469
nococcaceae (10.3%), and Porphyromonadaceae (6.92%). The 470
percentage of microorganisms not identified was 6.5%. The 471
other families as a whole had a relative abundance of 22.7%. At 472

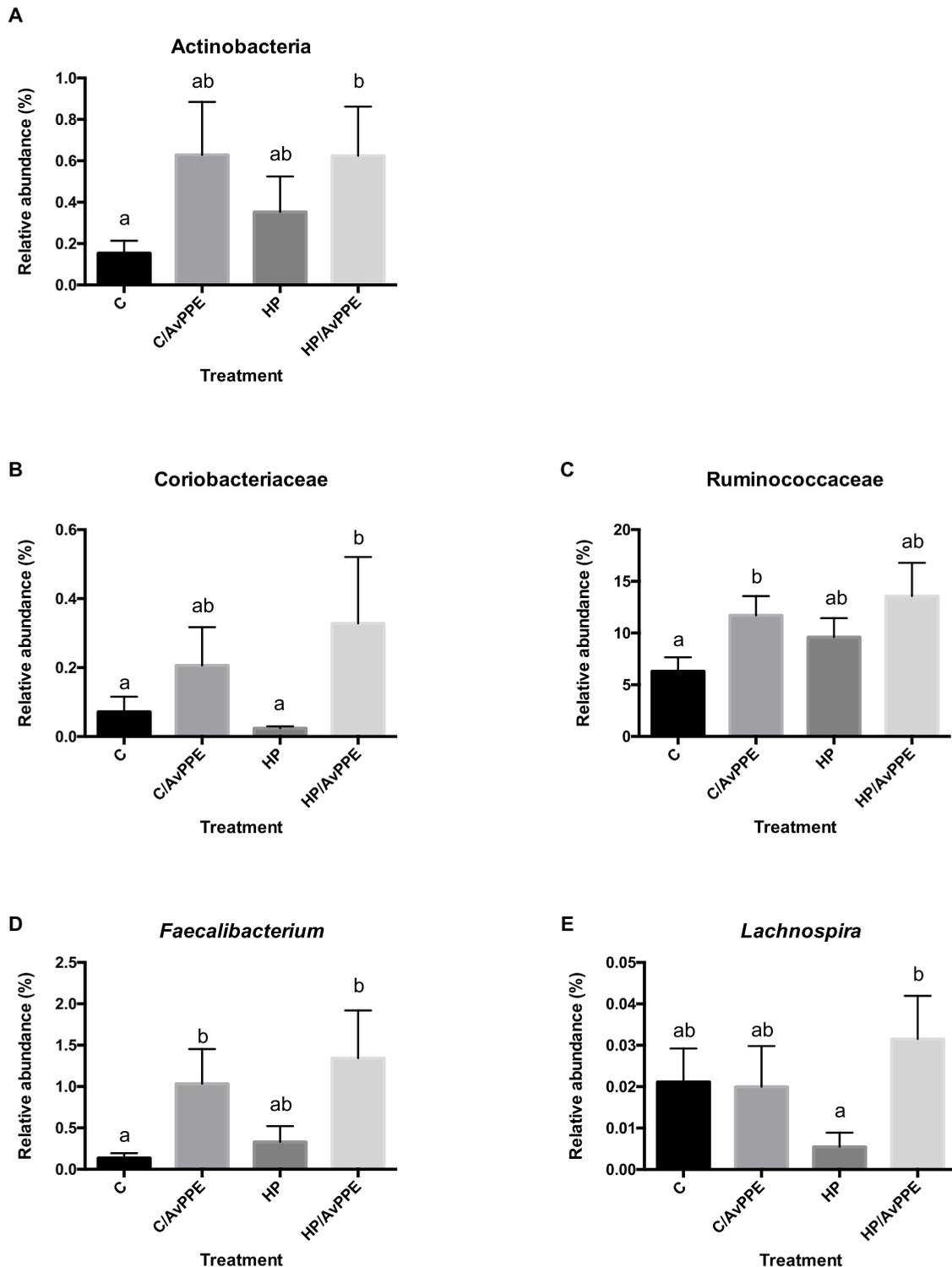


Figure 3. Relative abundance (%) of phyla, families, and genera of CSs that showed significant differences at T2. Each bar represents the average of five subjects (with SEM). Data were analyzed by Friedman's test with repeated measures ((A) $p = 0.02$; (B) $p = 0.006$; (C) $p = 0.02$; (D) $p < 0.001$;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

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

At the genus level, considering the average of the four
 480 treatments at T2, 33.9% of the microorganisms were not
 481 identified. *Bacteroides* (21.7%) was the most abundant genus,
 482 followed by *Parabacteroides* (6.9%), *Sutterella* (5.5%),
 483 [*Ruminococcus*] (4.4%), *Bilophila* (3.2%), [*Eubacterium*]
 484 (2.8%), and *Coprococcus* (2.6%). The other genera as a
 485 whole had a relative abundance of 8.3%. At T2, two genera, 486

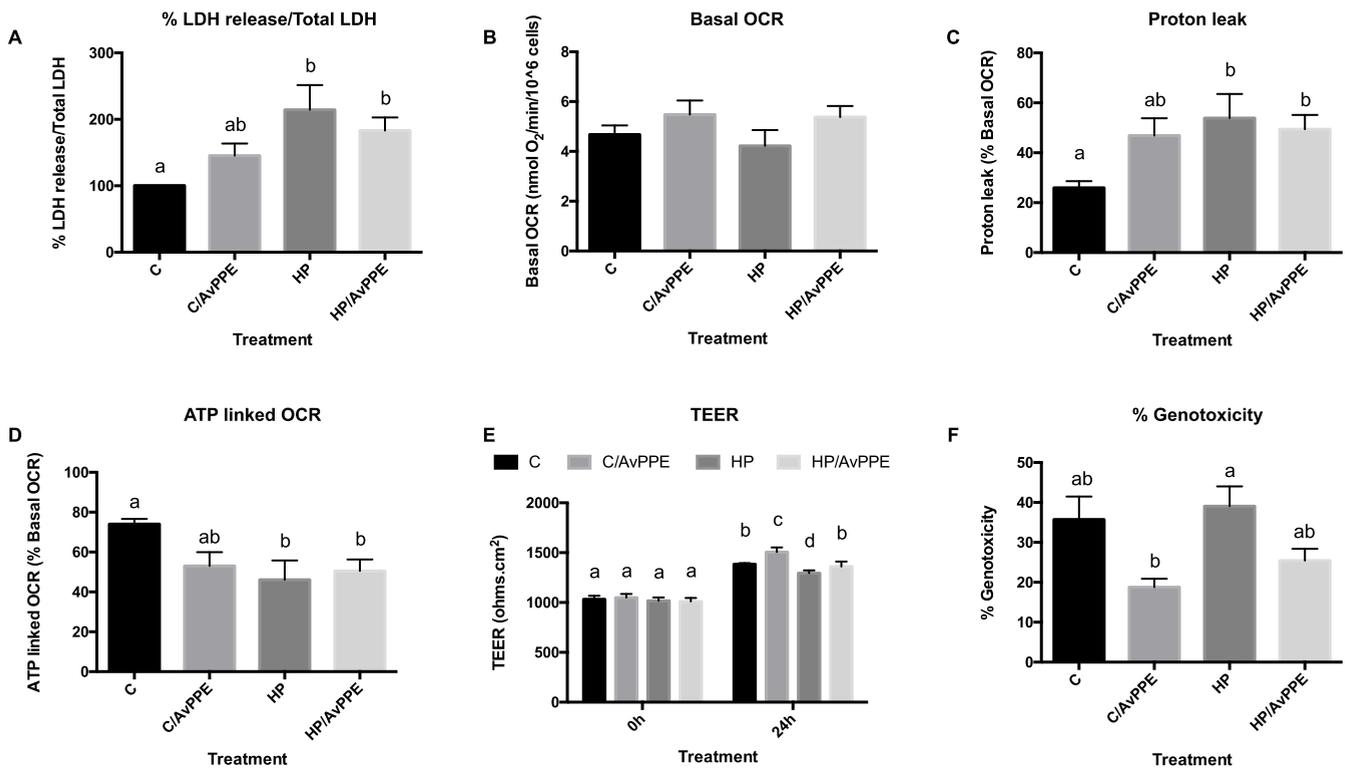


Figure 4. Effect of the CSs at T2 (10%, 72 h of incubation with daily replacement of the culture medium in HT-29Glc^{-/+} cells) on the (A) LDH release* in regard to intracellular total LDH, (B) basal oxygen consumption rate (OCR), (C) proton leak, and (D) ATP-linked OCR. (E) effect of the CSs (30%, 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.

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

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 *Butyricimonas*. 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).

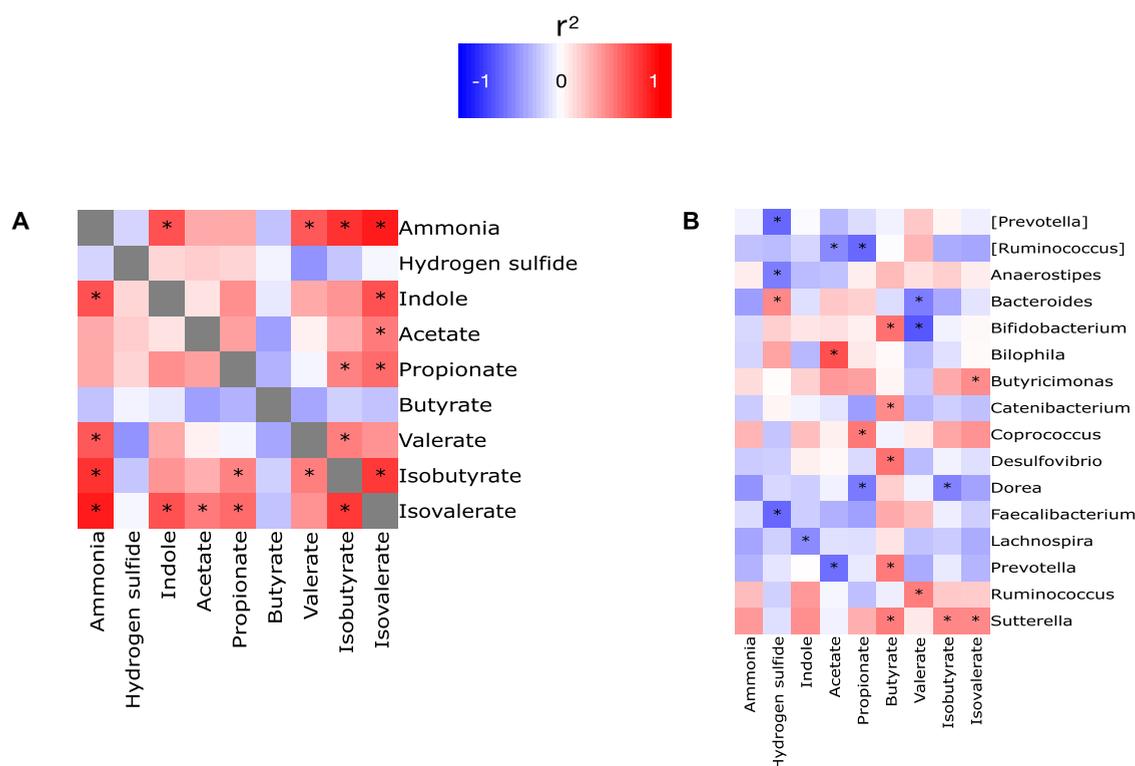


Figure 5. Heatmap of the correlations between the different parameters measured. All of the data were used for the analysis (all treatments for each parameter). The color indicates the value of the Spearman correlation coefficient. The asterisk indicates that the correlation is statistically significant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

542 ■ DISCUSSION

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

564 One of the selection criteria of our subjects was the presence
565 of SRB in their fecal microbiota, considering that these bacteria
566 produce H_2S , one of the compounds determined in our study.
567 SRB were detected in 66.7% of all subjects initially evaluated,
568 which is within the range of reported values for these bacteria
569 in human stools, both in prevalence and counts.³¹ H_2S in
570 excess is potentially toxic for the intestinal epithelium as it
571 inhibits colonocyte respiration, increases colonocyte gene
572 expression related to the inflammatory process, and has been

573 associated with a higher risk of ulcerative colitis and colorectal
574 cancer.^{14,20,32} The preventive effect shown by AvPPE against
575 the increased production of H_2S induced by HP was only
576 observed at T1. At T2, due to its volatility, H_2S was probably
577 eliminated during the culture period through the nitrogen flux
578 used to generate the anaerobic condition in the bioreactor, and
579 as a consequence, it could not be totally quantified. Our results
580 confirm a previous study reporting that quebracho PACs
581 reduced H_2S production when cultured with pig stools, these
582 latter effects being associated with an inhibition of the bacterial
583 metabolic activity and with a decrease in SRB and total
584 bacteria.³³ Similar observation was also reported with human
585 fecal microbiota cultured with a PAC-rich grape seed extract.³⁴
586 On the other hand, some dietary polyphenols have been shown
587 to remove volatile sulfur compounds in the mouth;³⁵
588 accordingly, it is possible that this phenomenon also occurs
589 in the colon with the avocado PACs, and this may explain the
590 protective effect shown by the AvPPE on H_2S production.

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

604 which could increase the indole-producing bacterial popula- 667
605 tions and/or their metabolic activity. In fact, AvPPE and HP 668
606 treatments, by themselves, tend to increase the concentrations 669
607 of indole in a nonsignificant manner when compared with C. 670
608 Regarding the SCFAs, these compounds are metabolic end 671
609 products of dietary fiber and, in less proportion, proteins. The 672
610 branched-chain SCFAs, isobutyrate and isovalerate, are 673
611 originated exclusively from amino acids and can be considered 674
612 as a marker of protein intake.¹⁴ The amount and type of SCFA 675
613 produced depend on the availability and type of substrates, the 676
614 composition of the IM, and the duration of intestinal transit.¹⁴

615 As expected, we observed increased concentrations of total 677
616 SCFA, uSCFA and bSCFA, with HP, as a result of microbial 678
617 fermentation and, more particularly, an increase of isobutyrate 679
618 and isovalerate. AvPPE partially prevented the increased SCFA 680
619 production induced by HP, suggesting that these compounds 681
620 interfere with bacterial protein metabolism. These latter results 682
621 are in accordance with the findings reported by Bazzocco et al. 683
622 using high-molecular-weight PACs from apple.³⁸ Interestingly, 684
623 the increase in propionate, valerate, isobutyrate, and isovalerate 685
624 concentrations induced by HP confirms the results of 686
625 Andriamihaja et al., in rats fed an HP diet (53% proteins) 687
626 for 2 weeks.³⁹ On the other hand, it is noteworthy that AvPPE 688
627 increases the levels of butyrate, an interesting result 689
628 considering the well-known health-promoting effects of this 690
629 SCFA.¹⁵

630 In general, all of the protein-derived metabolites correlated 691
631 positively between them except for butyrate. This phenom- 692
632 enon can be explained by the fact that butyrate is the only 693
633 metabolite that increases its concentration in the presence of 694
634 AvPPE, while the other metabolites increased with proteins.

635 We also studied the effect of the different treatments on the 695
636 composition of the fecal microbiota. Firmicutes and Bacter- 696
637 oidetes were the dominant phyla, as previously described.⁴⁰ 697
638 Interestingly, our results showed that, in the presence of 698
639 AvPPE, the abundance of the genus *Faecalibacterium* increased. 699
640 This genus includes the species *Faecalibacterium prausnitzii*, a 700
641 butyrate-producing microorganism that exerts anti-inflamma- 701
642 tory activities and whose prevalence is decreased in patients 702
643 with inflammatory bowel disease.⁴¹

644 Regarding the correlations, it is relevant to highlight the 703
645 positive correlation between H₂S and *Bacteroides* genus. It has 704
646 been described that bacteria of *Bacteroides* genus are capable to 705
647 synthesize sulfur volatile compounds like H₂S in the oral 706
648 cavity,⁴² and our result suggests that this phenomenon could 707
649 also occur in colon. On the other hand, H₂S was not correlated 708
650 with *Desulfovibrio*, the most representative genus of SRB in 709
651 human colon,¹⁸ suggesting that SRB are not the main H₂S 710
652 producer during the in vitro fermentation.³²

653 In the second step, we evaluated whether the exposure of 711
654 colonic epithelial cells to the CSs affected their viability, 712
655 mitochondrial function, barrier function and DNA integrity. 713
656 Our results show that the viability of HT-29Glc^{-/+} cells was 714
657 affected by the CS from HP, probably due to the presence of 715
658 protein bacterial metabolites. Accordingly, recently, Wong et 716
659 al., 2016, reported that HT-29Glc^{-/+} exposure to *p*-cresol, one 717
660 of these metabolites, induced cell damage and LDH release,⁸ 718
661 this deleterious effect being prevented by PAC-rich extracts 719
662 from fruits, including AvPPE. However, in the present study, 720
663 we did not observe any protective effect in the AvPPE-treated 721
664 group, maybe because the CSs contain several other 722
665 metabolites that could act synergistically, limiting the 723
666 protective effect of AvPPE. Additionally, if we consider that 724
725

we used a greater concentration of AvPPE in the culture 667
medium of the cells (10-fold more than the minimum 668
concentration used by Wong et al.), it is therefore possible 669
that such concentration induced a certain degree of toxicity 670
that could contribute to explain the lack of protective effect. 671
Regarding the mitochondrial function, we observed that cell 672
exposure to CSs from HP increased proton leak and decreased 673
ATP-linked OCR. This indicates that the energy efficiency in 674
treated cells is lower and that cell oxygen consumption is less 675
associated with ATP synthesis. This phenomenon could 676
contribute to the lower cell viability previously reported. At 677
the concentration assayed, AvPPE had no effect on these 678
altered parameters, even it seems somewhat toxic, thus 679
indicating that these compounds do not protect the cells 680
against viability loss. With respect to the intestinal barrier 681
function, Caco-2 cell monolayers exhibited a greater 682
permeability when exposed to CSs from HP, as reflected by 683
decreased TEER values. This is an important finding 684
considering that alterations in the gut barrier function generally 685
precede the development of inflammatory events at local and 686
systemic levels.⁸ Interestingly, CS from AvPPE treatment 687
reinforced barrier function (i.e., increased TEER values) 688
compared with CS from C, and in addition, it prevented the 689
barrier alterations induced by HP exposure. Similar results 690
have been reported with dietary polyphenols including PACs 691
in other models of intestinal barrier disturbances induced by 692
nonsteroidal anti-inflammatory drugs, bile salts, oxidants, or *p*- 693
cresol.^{43,44} Finally, we also determined the impact of CS 694
exposure on cell genotoxicity. Our results showed that CSs 695
from C/AvPPE and HP/AvPPE treatments attenuated 696
genotoxic damage, suggesting a potential antigenotoxic role 697
of AvPPE and confirming results from a previous study.⁴⁵ CS 698
from HP did not induce genotoxicity in our model, confirming 699
the study from Windey et al. carried out through the Comet 700
assay in HT-29 cells exposed to fecal water from subjects fed a 701
high-protein diet.⁴⁶ Similar results were reported by Beaumont 702
et al., where no DNA damage was observed in isolated 703
colonocytes of rats fed for 2 weeks with HP diets, assessed by 704
the Comet assay.⁴⁷ 705

In conclusion, our study confirms the deleterious effect of 706
HP diets on the colonic epithelium, with the increase of 707
protein-derived bacterial metabolites potentially toxic (like 708
ammonia and H₂S) and their negative effect on cell viability, 709
mitochondrial function, and intestinal barrier function of 710
intestinal epithelial cells. We also demonstrated that the 711
AvPPE exerted a protective effect in reducing the production 712
of ammonia and H₂S and increasing the production of butyrate 713
and indole, two beneficial metabolites for the colonic epithelia 714
and at systemic level. The AvPPE also prevented the 715
alterations in the intestinal permeability induced by HP 716
condition and increased the relative abundance of the butyrate- 717
producing genus *Faecalibacterium*. Finally, despite the 718
suggested toxic effect of AvPPE to cell viability and 719
mitochondrial function, the results of this work reveal a 720
promising compound with a healthy potential to prevent the 721
damage induced by protein metabolites and eventually HP 722
diets at the colonic level. To that, further investigation is 723
required, like adjusting the concentrations of the extract to 724
avoid toxic effects, both in in vitro studies and subsequently 725
animals and human studies. 726

727 ■ ASSOCIATED CONTENT

728 ● Supporting Information

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

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

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747 Notes

748 The authors declare no competing financial interest.

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753 participants of the study.

754 ■ ABBREVIATIONS

755 HPDs, high-protein diets; IM, intestinal microbiota; SCFA,
756 short-chain fatty acids; uSCFAs, unbranched short-chain fatty
757 acids; bSCFAs, branched short-chain fatty acids; H₂S,
758 hydrogen sulfide; PACs, proanthocyanidins; AvPPE, avocado
759 peel polyphenol extract; SRB, sulfate-reducing bacteria; C,
760 control; HP, hyperproteic or high-protein; CS, culture
761 supernatant; CSs, culture supernatants; NH₃, ammonia;
762 DMEM, Dulbecco's modified Eagle's medium; LDH, lactate
763 dehydrogenase; OCR, oxygen consumption rate; TEER,
764 transepithelial electrical resistance; OTUs, operational taxo-
765 nomic units; SEM, standard error of the mean

766 ■ REFERENCES

767 (1) Yahia, E. M.; Maldonado Celis, M. E.; Svendsen, M. The
768 Contribution of Fruit and Vegetable Consumption to Human Health.
769 *Fruit Veg. Phytochem.* **2017**, 1–52.
770 (2) Tremocoldi, M. A.; Rosalen, P. L.; Franchin, M.; Massarioli, A.
771 P.; Denny, C.; Daiuto, E. R.; Paschoal, J. A. R.; Melo, P. S.; de
772 Alencar, S. M. Exploration of avocado by-products as natural sources
773 of bioactive compounds. *PLoS One* **2018**, 13, No. e0192577.
774 (3) Figueroa, J. G.; Borrás-Linares, I.; Lozano-Sánchez, J.; Segura-
775 Carretero, A. Comprehensive identification of bioactive compounds
776 of avocado peel by liquid chromatography coupled to ultra-high-
777 definition accurate-mass Q-TOF. *Food Chem.* **2018**, 245, 707–716.
778 (4) Figueroa, J. G.; Borrás-Linares, I.; Lozano-Sánchez, J.; Segura-
779 Carretero, A. Comprehensive characterization of phenolic and other
780 polar compounds in the seed and seed coat of avocado by HPLC-
781 DAD-ESI-QTOF-MS. *Food Res. Int.* **2018**, 105, 752–763.
782 (5) Rodrigo, R.; Libuy, M.; Feliu, F.; Hasson, D. Polyphenols in
783 disease: from diet to dupplements. *Curr. Pharm. Biotechnol.* **2014**, 15,
784 304–317.

(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. 788

(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. *791*
Annu. Rev. Food Sci. Technol. **2016**, 7, 43–64. 792

(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. 798

(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. 803

(10) Clifton, P. M.; Keogh, J. Metabolic effects of high-protein diets.
Curr. Atheroscler. Rep. **2007**, 9, 472–478. 805

(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. 808

(12) Windey, K.; De Preter, V.; Verbeke, K. Relevance of protein
fermentation to gut health. *Mol. Nutr. Food Res.* **2012**, 56, 184–196. 810

(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. 813

(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. 817

(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. 820

(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. 826

(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. 829

(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. 832

(19) Tzounis, X.; Vulevic, J.; Kuhnle, G. G.; George, T.; Leonczak,
J.; Gibson, G. R.; Kwik-Urbe, C.; Spencer, J. P. Flavanol monomer-
induced changes to the human faecal microflora. *Br. J. Nutr.* **2008**, 99,
782–792. 836

(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. 842

(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. 846

(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. 849

(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 chromatog-
raphy. *Biomed. Chromatogr.* **2006**, 20, 674–682. 853

- 854 (24) Fujio-Vejar, S.; Vasquez, Y.; Morales, P.; Magne, F.; Vera-Wolf,
855 P.; Ugalde, J. A.; Navarrete, P.; Gotteland, M. The Gut Microbiota of
856 Healthy Chilean Subjects Reveals a High Abundance of the Phylum
857 Verrucomicrobia. *Front. Microbiol.* **2017**, *8*, No. 1221.
- 858 (25) Caporaso, J. G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.;
859 Bushman, F. D.; Costello, E. K.; Fierer, N.; Pena, A. G.; Goodrich, J.
860 K.; Gordon, J. I.; Huttley, G. A.; Kelley, S. T.; Knights, D.; Koenig, J.
861 E.; Ley, R. E.; Lozupone, C. A.; McDonald, D.; Muegge, B. D.;
862 Pirrung, M.; Reeder, J.; Sevinsky, J. R.; Tumbaugh, P. J.; Walters, W.
863 A.; Widmann, J.; Yatsunenko, T.; Zaneveld, J.; Knight, R. QIIME
864 allows analysis of high-throughput community sequencing data. *Nat.*
865 *Methods* **2010**, *7*, 335–336.
- 866 (26) McDonald, D.; Price, M. N.; Goodrich, J.; Nawrocki, E. P.;
867 DeSantis, T. Z.; Probst, A.; Andersen, G. L.; Knight, R.; Hugenholtz,
868 P. An improved Greengenes taxonomy with explicit ranks for
869 ecological and evolutionary analyses of bacteria and archaea. *ISME*
870 *J.* **2012**, *6*, 610–618.
- 871 (27) Legrand, C.; Bour, J. M.; Jacob, C.; Capiamont, J.; Martial, A.;
872 Marc, A.; Wudtke, M.; Kretzmer, G.; Demangel, C.; Duval, D.; Hache,
873 J. Lactate dehydrogenase (LDH) activity of the number of dead cells
874 in the medium of cultured eukaryotic cells as marker. *J. Biotechnol.*
875 **1992**, *25*, 231–243.
- 876 (28) Carrasco-Pozo, C.; Tan, K. N.; Reyes-Farias, M.; De La Jara,
877 N.; Ngo, S. T.; Garcia-Diaz, D. F.; Llanos, P.; Cires, M. J.; Borges, K.
878 The deleterious effect of cholesterol and protection by quercetin on
879 mitochondrial bioenergetics of pancreatic beta-cells, glycemic control
880 and inflammation: In vitro and in vivo studies. *Redox Biol.* **2016**, *9*,
881 229–243.
- 882 (29) Andriamihaja, M.; Lan, A.; Beaumont, M.; Audebert, M.;
883 Wong, X.; Yamada, K.; Yin, Y. L.; Tome, D.; Carrasco-Pozo, C.;
884 Gotteland, M.; Kong, X. F.; Blachier, F. The deleterious metabolic
885 and genotoxic effects of the bacterial metabolite *p*-cresol on colonic
886 epithelial cells. *Free Radical Biol. Med.* **2015**, *85*, 219–227.
- 887 (30) Hassanat, F.; Benchaar, C. Assessment of the effect of
888 condensed (acacia and quebracho) and hydrolysable (chestnut and
889 valonea) tannins on rumen fermentation and methane production in
890 vitro. *J. Sci. Food Agric.* **2013**, *93*, 332–339.
- 891 (31) Stewart, J. A.; Chadwick, V. S.; Murray, A. Carriage,
892 quantification, and predominance of methanogens and sulfate-
893 reducing bacteria in faecal samples. *Lett. Appl. Microbiol.* **2006**, *43*,
894 58–63.
- 895 (32) Blachier, F.; Beaumont, M.; Kim, E. Cysteine-derived hydrogen
896 sulfide and gut health: a matter of endogenous or bacterial origin.
897 *Curr. Opin. Clin. Nutr. Metab. Care* **2019**, *22*, 68–75.
- 898 (33) Whitehead, T. R.; Spence, C.; Cotta, M. A. Inhibition of
899 hydrogen sulfide, methane, and total gas production and sulfate-
900 reducing bacteria in in vitro swine manure by tannins, with focus on
901 condensed quebracho tannins. *Appl. Microbiol. Biotechnol.* **2013**, *97*,
902 8403–8409.
- 903 (34) Yamakoshi, J.; Tokutake, S.; Kikuchi, M.; Kubota, Y.; Konishi,
904 H.; Mitsuoka, T. Effect of Proanthocyanidin-Rich Extract from Grape
905 Seeds on Human Fecal Flora and Fecal Odor. *Microb. Ecol. Health Dis.*
906 **2001**, *13*, 25–31.
- 907 (35) Zeng, Q. C.; Wu, A. Z.; Pika, J. The effect of green tea extract
908 on the removal of sulfur-containing oral malodor volatiles in vitro and
909 its potential application in chewing gum. *J. Breath Res.* **2010**, *4*,
910 No. 036005.
- 911 (36) Bansal, T.; Alaniz, R. C.; Wood, T. K.; Jayaraman, A. The
912 bacterial signal indole increases epithelial-cell tight-junction resistance
913 and attenuates indicators of inflammation. *Proc. Natl. Acad. Sci. U.S.A.*
914 **2010**, *107*, 228–233.
- 915 (37) Chimere, C.; Emery, E.; Summers, D. K.; Keyser, U.; Gribble,
916 F. M.; Reimann, F. Bacterial metabolite indole modulates incretin
917 secretion from intestinal enteroendocrine L cells. *Cell Rep.* **2014**, *9*,
918 1202–1208.
- 919 (38) Bazzocco, S.; Mattila, I.; Guyot, S.; Renard, C. M.; Aura, A. M.
920 Factors affecting the conversion of apple polyphenols to phenolic
921 acids and fruit matrix to short-chain fatty acids by human faecal
922 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. 923
Colon luminal content and epithelial cell morphology are markedly 924
modified in rats fed with a high-protein diet. *Am. J. Physiol.: 925*
Gastrointest. Liver Physiol. **2010**, *299*, G1030–G1037. 926
- (40) Eckburg, P. B.; Bik, E. M.; Bernstein, C. N.; Purdom, E.; 928
Dethlefsen, L.; Sargent, M.; Gill, S. R.; Nelson, K. E.; Relman, D. A. 929
Diversity of the human intestinal microbial flora. *Science* **2005**, *308*, 930
1635–1638. 931
- (41) Miquel, S.; Martín, R.; Rossi, O.; Bermúdez-Humarán, L. G.; 932
Chatel, J. M.; Sokol, H.; Thomas, M.; Wells, J. M.; Langella, P. 933
Faecalibacterium prausnitzii and human intestinal health. *Curr. Opin. 934*
Microbiol. **2013**, *16*, 255–261. 935
- (42) Tonzetich, J.; McBride, B. C. Characterization of volatile 936
sulphur production by pathogenic and non-pathogenic strains of oral 937
Bacteroides. *Arch. Oral Biol.* **1981**, *26*, 963–969. 938
- (43) Carrasco-Pozo, C.; Morales, P.; Gotteland, M. Polyphenols 939
protect the epithelial barrier function of Caco-2 cells exposed to 940
indomethacin through the modulation of occludin and zonula 941
occludens-1 expression. *J. Agric. Food Chem.* **2013**, *61*, 5291–5297. 942
- (44) Erlejman, A. G.; Fraga, C. G.; Oteiza, P. I. Procyanidins protect 943
Caco-2 cells from bile acid- and oxidant-induced damage. *Free Radical 944*
Biol. Med. **2006**, *41*, 1247–1256. 945
- (45) Bakheet, S. A.; Alhuraishi, A. M.; Al-Harbi, N. O.; Al-Hosaini, 946
K. A.; Al-Sharary, S. D.; Attia, M. M.; Alhoshani, A. R.; Al-Shabanah, 947
O. A.; Al-Harbi, M. M.; Imam, F.; Ahmad, S. F.; Attia, S. M. 948
Alleviation of Aflatoxin B1-Induced Genomic Damage by Proantho- 949
cyanidins via Modulation of DNA Repair. *J. Biochem. Mol. Toxicol.* 950
2016, *30*, 559–566. 951
- (46) Windey, K.; De Preter, V.; Louat, T.; Schuit, F.; Herman, J.; 952
Vansant, G.; Verbeke, K. Modulation of Protein Fermentation Does 953
Not Affect Fecal Water Toxicity: A Randomized Cross-Over Study in 954
Healthy Subjects. *PLoS One* **2012**, *7*, No. e52387. 955
- (47) Beaumont, M.; Andriamihaja, M.; Armand, L.; Grauso, M.; 956
Jaffrézic, F.; Laloë, D.; Moroldo, M.; Davila, A.-M.; Tomé, D.; 957
Blachier, F.; Lan, A. Epithelial response to a high-protein diet in rat 958
colon. *BMC Genomics* **2017**, *18*, No. 116. 959