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Effect of a proanthocyanidin-rich polyphenol extract from avocado on the production of amino acid-derived bacterial metabolites and the microbiota composition in rats fed a high-protein diet†

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The consumption of high-protein diets (HPDs) increases the flux of undigested proteins moving to the colon. These proteins are hydrolyzed by bacterial proteases and peptidases, releasing amino acids, which in turn are metabolized by the intestinal microbiota (IM) for protein synthesis and production of various metabolites that can exert positive or deleterious effects, depending on their concentrations, at the colonic or systemic level. On the other hand, proanthocyanidins are polymers of flavan-3-ols which cannot be absorbed at the intestinal level, accumulating in the colon where they are fermented by the IM producing metabolites that appear beneficial for colonocytes and also at the peripheral level. This study evaluated the effect of an avocado peel polyphenol extract (AvPPE) rich in proanthocyanidins on the production of cecal bacterial metabolites and microbiota composition in rats fed a HPD. Compared with the normal-protein (NP) group, HPD did not markedly affect the body weight gain of the animals, but increased the kidney weight. Additionally, the HPD induced a higher cecal concentration of ammonia (NH_4^+/NH_3), hydrogen sulfide (H_2S) and branched-chain fatty acids (BCFAs). The supplementation with AvPPE attenuated the production of H_2S and increased the production of indole. On the other hand, the HPD affected the composition of the cecal microbiota, increasing the relative abundance of the genera *Bacteroides* and *Lactobacillus*, while decreasing *Prevotella*. The AvPPE counteracted the increase induced by the HPD on the genus *Lactobacillus*, and increased the relative abundance of [*Prevotella*]. Our results contribute towards explaining the health-promoting effects of proanthocyanidin-rich dietary foodstuffs including fruits and vegetables.

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1 Introduction

High-protein diets (HPDs) provide almost twice as much protein as normal diets (25–35% *vs.* 12–18% of energy intake).[1] They are commonly used to increase the muscular mass or to lose body weight, mainly due to their thermogenic and satiating effects.[1–4] Even if most of the dietary proteins are hydrolyzed and absorbed in the small intestine, part of them (which is higher in subjects consuming HPDs) reaches the colon in an undigested form or as peptides that are hydrolyzed by bacterial proteases, releasing amino acids subsequently used for bacterial protein synthesis and biomass growth.[5,6] These amino acids can also be fermented by the intestinal microbiota (IM), generating bacterial metabolites such as short-chain fatty acids (SCFAs) and indole, which are considered beneficial for the colonic epithelia and at the systemic

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level, and other potentially toxic compounds such as ammonia (considered as the sum of NH_4^+ and NH_3), *p*-cresol and hydrogen sulfide (H_2S), which can impair the energy supply through an inhibition of the colonocyte respiration, among other effects.[5–10]

On the other hand, proanthocyanidins (PACs) or condensed tannins are complex polymers of flavan-3-ols with a degree of polymerization between 3 and 30 units (in oligomeric fractions) or >30 (in polymeric fractions), and are abundant in a number of fruits and vegetables and plant-derived foodstuffs.[11–14] Animal and human studies suggest that PAC intake is associated with a lower risk of cancer, cardiovascular disease and diabetes.[15,16] Only PAC monomers and dimers can be absorbed in the small intestine, while oligo- and polymers of a higher degree of polymerization accumulate in the intestinal lumen and are transferred to the colon.[13,17] Accordingly, it may be stated that the gastrointestinal tract is a key organ for the healthy effects of PACs.[18] In the colon, PACs are metabolized by the IM,[13,17] resulting in the formation of a great array of bacterial metabolites, including aromatic acids and valerolactones, which can be absorbed by the colonocytes and pass to the bloodstream to eventually exert their beneficial activities in other tissues and organs.[11,13,17] It is currently postulated that most of the beneficial effects of PACs are largely due to these metabolites.[11] We recently reported that PAC-rich extracts from apple, grape, avocado and cranberry as well as microbiota-derived PAC metabolites prevent, *in vitro*, the deleterious effect induced by *p*-cresol[19] and hydrogen sulfide[20] on intestinal/colonic cell lines, suggesting that these dietary bioactive compounds could protect the colonic epithelia against the negative effects induced by HPDs.

Additionally, existing evidence shows that both undigested proteins and PACs can modulate the IM composition. The intake of high-protein, low-carbohydrate diets are associated with a more pathogenic and pro-inflammatory profile of the microbiota with increased populations of *Clostridium, Enterococcus*, *Shigella*, *Escherichia coli* and *Bacteroides*, and decreased populations of *Bifidobacterium*, *Roseburia* and *Eubacterium rectale*.[21] On the other hand, PAC intake is associated with higher levels of beneficial microorganisms such as *Bifidobacterium*, *E. coli*, *Lactobacillus*, *Enterococcus*, *Akkermansia* and the *C. coccoides–E. rectale* group, and lower levels of *C. histolyticum*.[22–26]

Considering this context, the aim of the current study was to evaluate whether the regular administration of a well characterized PAC-rich polyphenol extract from avocado peel prevented the increased production of deleterious amino acid-derived bacterial metabolites and the changes in the cecal microbiota composition in rats fed with a HPD.

2 Materials and methods

2.1 Avocado peel polyphenol extract (AvPPE)

Fresh avocados (*Persea americana* Mill., Hass variety) were purchased from a local market and their peels were used for the elaboration of the AvPPE, as previously described.[19,27] Briefly, 500 g of frozen peels were mixed with hot water and filtered on a sintered glass funnel. Marcs were homogenized and extracted again with hot water. The pooled extracts were adjusted to pH 2.5 and poured on a Sepabeads SP-850-packed glass column pre-conditioned with acidic water. Sugars and other water-soluble compounds were removed by washing with distilled water and the polyphenol extract was obtained after elution with absolute ethanol. Dried polyphenol-rich extracts were stored at −70 °C until use. The identification and quantification of the polyphenols present in the extract were carried out by RP-HPLC using a Waters Alliance 2695 system equipped with a C18 column and a C18 pre-column. Quantification was performed by using UV-VIS chromatograms acquired at 350 nm for flavonols, 320 nm for phenolic acids, 280 nm for flavan-3-ols and 520 nm for anthocyanins. Standard compounds were used to construct the respective external calibration curves. The polyphenolic content of the extract is described in Table 1. Globally, it contained 14% flavan-3-ol monomers, 29.1% PACs (types A and B, only composed of epicatechin, with a mean degree of polymerization of 6.1), 15.1% flavonoids, 3% anthocyanins, and 38.8% phenolic acids.

2.2 Animals and experimental procedure

Forty male Wistar rats 5 weeks of age were assigned to one of the four following groups of treatment (n = 10): Control (C, 15% protein); Control + AvPPE (300 mg kg^{-1}) (C/AvPPE); High-protein (HP, 55% protein) and High-protein + AvPPE (HP/AvPPE). The Control diet (D10012M: 15% protein, 76% carbohydrates and 9% fat) and the HPD (a modification of the D10012M diet containing 55% protein, 36% carbohydrates and 9% fat) were purchased from Research Diets (New Jersey, USA). Both diets were isocaloric, used casein as the source of protein, and had the same amount of fiber (cellulose, 50 g kg^{-1}). Table 2 summarizes the nutritional compositions of both the diets. The AvPPE was administered daily with the diet at a dose of 300 mg per kg of body weight, corresponding to 90 mg of PACs per kg. This dose was calculated using the body surface area (BSA) normalization method to convert human to animal doses,[28] considering a human consumption of 850 mg PACs day^{-1} as a forced model of high PAC consumption, taking into account the fact that the normal consumption ranged between 100 and 500 mg PACs day^{-1}.[13] Litters from sister mothers were selected because they show less variability in the composition of their IM.[29] Rats were maintained for 1 week of acclimatization consuming the control diet and subsequently, they received the different diets for 4 weeks. The animals were weighed twice a week during the dietary intervention period. At the end of this period, they were euthanized by decapitation under anesthesia with ketamine/xylazine. The colon, cecum, liver, spleen and kidneys were extracted from the animals and weighed. The cecal content was aliquoted in separate tubes for the different analyses. The organs and aliquoted cecal samples were frozen at −80 °C until analysis. This study was performed in accordance with the Guide for the care and use of laboratory animals, National Research

Table 1 Phenolic composition of the AvPPE (mean ± SD)

Compound	% in AvPPE
Flavan-3-ol monomers	
Epicatechin	12.09 ± 0.38
Catechin	1.92 ± 0.01
Epicatechin gallate	ND
Catechin gallate	ND
∑Flavan-3-ol monomers	**14.01 ± 0.20**
∑Total procyanidins	**29.08 ± 1.01**
Flavonoids	
Hyperoside	ND
Isoquercitrin	ND
Quercitrin	ND
Quercetin	1.23 ± 0.02
Quercetin-*O*-pentosides (≠of rutin)	ND
Rutin	1.09 ± 0.04
Apigenin	12.80 ± 0.12
Kaempferol	ND
Kaempferol derivatives	ND
Myricetin	ND
Myricetin hexosides	ND
Isorhamnetin	ND
Isorhamnetin derivatives	ND
∑Flavonoids	**15.12 ± 0.06**
Anthocyanins	
Cyanidin-*O*-hexosides	ND
Cyanidin-*O*-arabinoside	ND
Cyanidin-3-*O*-glucoside	3.00 ± 0.03
Peonidin-*O*-hexosides	ND
Peonidin-*O*-arabinoside	ND
Malvidin-*O*-hexosides	ND
Delphinidin-*O*-hexosides	ND
Petunidin-*O*-hexosides	ND
∑Total anthocyanins	**3.00 ± 0.03**
Phenolic acids	
Gallic acid	ND
Syringic acid	ND
Vanillic acid	3.16 ± 0.01
Chlorogenic acid	1.00 ± 0.02
Caffeic acid	8.01 ± 0.01
Ellagic acid	ND
Sinapic acid	0.25 ± 0.02
Ferulic acid	6.54 ± 0.02
Anisic acid	2.28 ± 0.02
p-Coumaric acid	ND
Cinnamic acid	ND
5-Caffeoyl quinic acid	ND
4-Caffeoyl quinic acid	ND
Protocatechuic acid	0.98 ± 0.09
p-Hydroxybenzoic acid	9.92 ± 0.08
m-Hydroxybenzoic acid	6.65 ± 0.10
Caffeoyl glucoside	ND
Feruloyl glucoside	ND
Coumaroyl glucoside	ND
∑Phenolic acids	**38.79 ± 0.04**
Dihydrochalcones	
Phloretin-2′-glucoside	ND
Phloretin-2′-xyloglucoside	ND
∑Dihydrochalcones	**ND**
Total	100 ± 0.27

ND = not detected.

Table 2 Compositions of the experimental diets

Ingredient (g kg^{-1})	Control	High-protein
Casein	140	522
L-Cystine	1.8	7
Corn starch	495.7	108.5
Maltodextrin 10	125	125
Sucrose	100	100
Cellulose, BW200	50	50
Soybean oil	40	40
t-Butylhydroquinone	0.008	0.008
Mineral mix S10022M	35	35
Vitamin mix V10037	10	10
Choline bitartrate	2.5	2.5
Energy (kcal)	3850	3850

Council of The National Academies (USA), 8th Edition, 2011 and was approved by the Bioethics Committee in Animal Research of the Faculty of Medicine of the University of Chile (No. 0704).

2.3 Determination of amino acid-derived bacterial metabolites in the cecal content

2.3.1 Ammonia (NH_4^+/NH_3). The cecal contents (100 mg) were diluted 10 fold with deionized water, vortexed, centrifuged at 14 000*g* for 15 min at 4 °C, and the supernatants were deproteinized with Microcon centrifugal filters, 10 kDa (Merck Millipore, Santiago, Chile). Ammonia determination was carried out by using a commercial kit (K-AMIAR 07/14, Megazyme, Chicago, USA), following the manufacturer's recommendations. Deproteinized samples were diluted 1.5 fold with deionized water and NH_4^+/NH_3 was determined at 340 nm in 96 well plates as a result of its reaction with 2-oxoglutarate to form L-glutamic acid and $NADP^+$ in the presence of glutamate dehydrogenase and NADPH. The consumption of NADPH during the reaction was quantified by monitoring the absorbance changes at 340 nm.

2.3.2 Hydrogen sulfide (H_2S). Hydrogen sulfide was measured by GC-MS after sulfide alkylation following the methodology previously described.[10] 100 mg of the cecal content were weighed (avoiding sample defrosting) and mixed with 100 μL of 5 mM benzalkonium chloride (prepared in deoxygenated Milli-Q water saturated with sodium tetraborate), 100 μL of 20 mM pentafluorobenzylbromide (in toluene) and 100 μL of ethyl acetate (containing 100 μM naphthalene as an internal standard). The mixture was then shaken at 55 °C for 4 h. Thereafter, 150 μL of KH_2PO_4 (saturated in Milli-Q water) were added, the mixture was vortexed for 1 min and centrifuged (10 000*g*, 10 min, 4 °C) and the organic phase was removed and analyzed by GC-MS. A calibration curve was plotted with standard Na_2S solutions between 12.5 and 100 μM. Samples and standard solutions (1 μL) were injected into an Agilent 6890N gas chromatograph coupled to a 5973N mass spectrometer (Agilent Technologies, USA) equipped with a capillary column HP-5HS (30 m length, 0.25 mm internal diameter, coated with a 0.25 μm film) (Agilent, USA). Chromatographic conditions were defined as previously

described.[10] The area under the curve of the samples and the standard solutions was corrected by the area under the curve of the internal standard and the results were expressed as μM.

2.3.3 Indole. Indole was determined using the method described by Darkoh *et al.*, 2015.[30] Briefly, 250 mg of the cecal content were weighed, mixed with 750 μL of 70% ethanol, vortexed and then incubated for 10 min at 70 °C in a thermo-regulated water bath. Samples were then vortexed and centrifuged (20 min, 40 °C, 14 000 rpm), and the supernatants were diluted 5 fold with 70% ethanol. A calibration curve was plotted with an indole standard prepared in 70% ethanol in a range of concentrations between 0 and 300 μM. One hundred microliters of the samples or standards were mixed with 25 μL of 5.3 M NaOH and 50 μL of 0.3 M hydroxylamine hydrochloride (NH_2OH–HCl) in a 96 well plate. After 15 min incubation at room temperature (≈25 °C), 125 μL of 2.7 M H_2SO_4 were added, the plate was incubated for 30 min at room $T°$ and the absorbance was measured at 530 nm in an Infinite 200 PRO plate reader (TECAN, Switzerland).

2.3.4 Short-chain fatty acids (SCFAs). Short-chain fatty acid (SCFA) and branched-chain fatty acid (BCFA) determination was carried out by gas chromatography as previously described by Zhao *et al.*,[31] with some modifications. 200 mg of the cecal content were mixed with 1 mL of Milli-Q water, vortexed for 1 min, and the pH of the suspension was adjusted at 2–3 with 0.68 M HCl. The samples were then incubated for 10 min at room $T°$ with occasional agitation, centrifuged (10 min, 14 000 rpm, room $T°$) and 2-ethyl butyric acid as an internal standard was added at a final concentration of 1 mM. The SCFA detection and quantification were performed in an Agilent 7890A gas chromatograph (Agilent, USA) equipped with a FID detector and a Restek Stabilwax-Da (fused silica) capillary column (Restek, USA) (30 m length, 0.32 mm internal diameter). The separation of the SCFAs was carried out between 120 and 240 °C, with an initial heating at 120 °C for 1 min, an increase to 240 °C at a rate of 10 °C min^{-1} and a 3 min standby at 240 °C. Hydrogen was used as the gas carrier, the $T°$ of the injector and detector was 240 °C and 265 °C, respectively. A mix of acetic acid, propionic acid, butyric acid, valeric acid, isobutyric acid and isovaleric acid (1 mg mL^{-1} each, Restek, USA) was used for calibration. The area under the curve of the samples and standard solutions was corrected by the area under the curve of the internal standard, and the results were expressed as mM.

2.4 Determination of the composition of the cecal microbiota

The bacterial DNA from the cecal content was extracted with a QIAamp Fast DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions, with a supplementary step of mechanical lysis with a bead beater. The extracted DNA was sent to the Roy J. Carver Biotechnology Center (University of Illinois) to determine the microbiota composition following the same methodology described by Fujio-Vejar *et al.*, 2017.[32] The region V3–V4 of the 16S rRNA gene was amplified by PCR and the amplicons were subsequently sequenced using the Illumina MiSeq platform. The sequencing service of the W.M. Keck Center for Comparative and Functional Genomics (University of Illinois) delivered demultiplexed and barcode depleted sequences. Paired-end sequences were joined using PEAR[33] and primer sequences were depleted using a fastx-trimmer from the FASTX-Toolkit. To analyze the 16S rRNA gene sequences, the QIIME software (V1.9.1) was used.[34,35] Using an open-reference command, operational taxonomic units (OTUs) were selected and were then defined by clustering at 3% divergence (97% similarity) using as a reference the GreenGenes database[36,37] release 08-2013. Results were expressed as the relative abundance.

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

2.5 Statistical analysis

The variables were expressed as the mean and standard error of the mean (SEM). The normal distribution of the variables was determined using the Shapiro Wilks test and, depending on the results, means were compared through a parametric test (one way ANOVA and Tukey *post hoc* test) or no parametric test (Kruskal–Wallis and Dunn *post hoc* test). The correlations between the different parameters were analyzed using the Spearman's correlation coefficient. Statistical significance was considered with a $p < 0.05$. The statistical software GraphPad Prism version 6.0 was used for the analysis.

3 Results

3.1 Body and organ weight

No difference was observed in body weights among the different treatment groups (Fig. 1A). As shown in Fig. 1B and C, liver and spleen weights (in % of the total body weight) at the end of the treatment period did not differ between control and treated animals, while the kidney relative weights were significantly higher in the HP and HP/AvPPE groups, compared with the C and AvPPE groups (Fig. 1D).

3.2 Changes in cecal bacterial metabolites

Compared with C, the cecal concentration of ammonia significantly increased in animals that consumed the HP diet, and the concomitant intake of the extract (HP/AvPPE) did not prevent this increase (Fig. 2A). Regarding the cecal concentration of H_2S, it significantly increased in the HP group, compared with C, and this phenomenon was prevented in the HP/AvPPE group (Fig. 2B). As shown in Fig. 2C, the cecal concentration of indole increased in the animals that consumed the C/AvPPE diet, compared with C, while this parameter was not affected in the rats fed the HP diet. Additionally, the cecal indole concentration was significantly lower under the HP conditions compared with those under C/AvPPE and HP/AvPPE conditions.

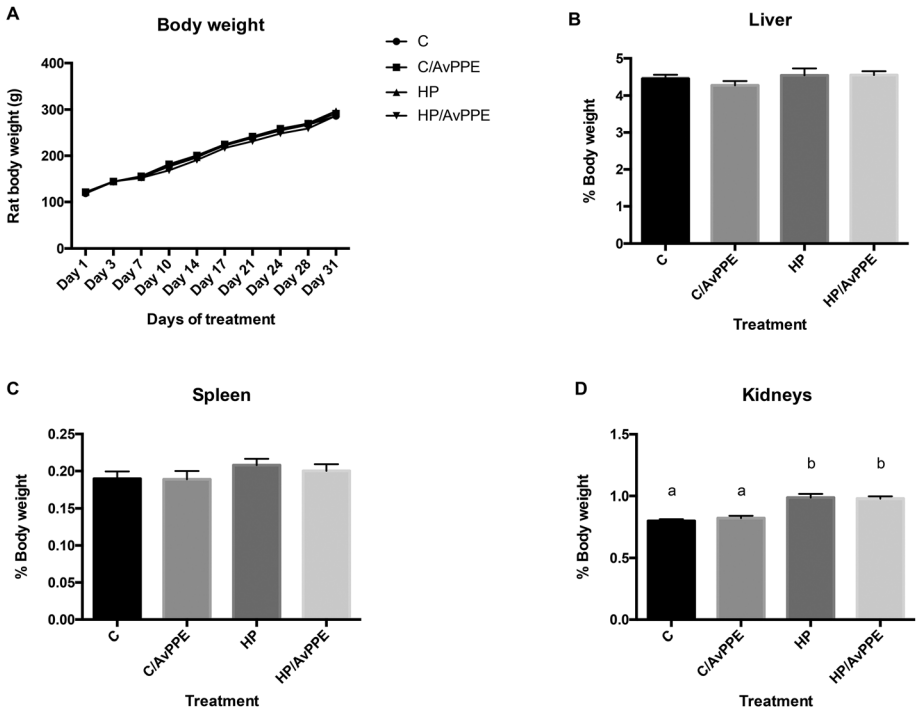

Fig. 1 Body weight curve (A), weights of the liver (B), spleen (C) and kidneys (D) of rats fed for 4 weeks with the different diets, with or without AvPPE supplementation. Each bar represents the average of 10 rats (with SEM). Data were analyzed by one-way ANOVA (p = 0.9929 (A), p = 0.4496 (B), p = 0.4481 (C), p < 0.0001 (D)) and the Tukey *post-hoc* test. Bars with different letters are significantly different (p < 0.05).

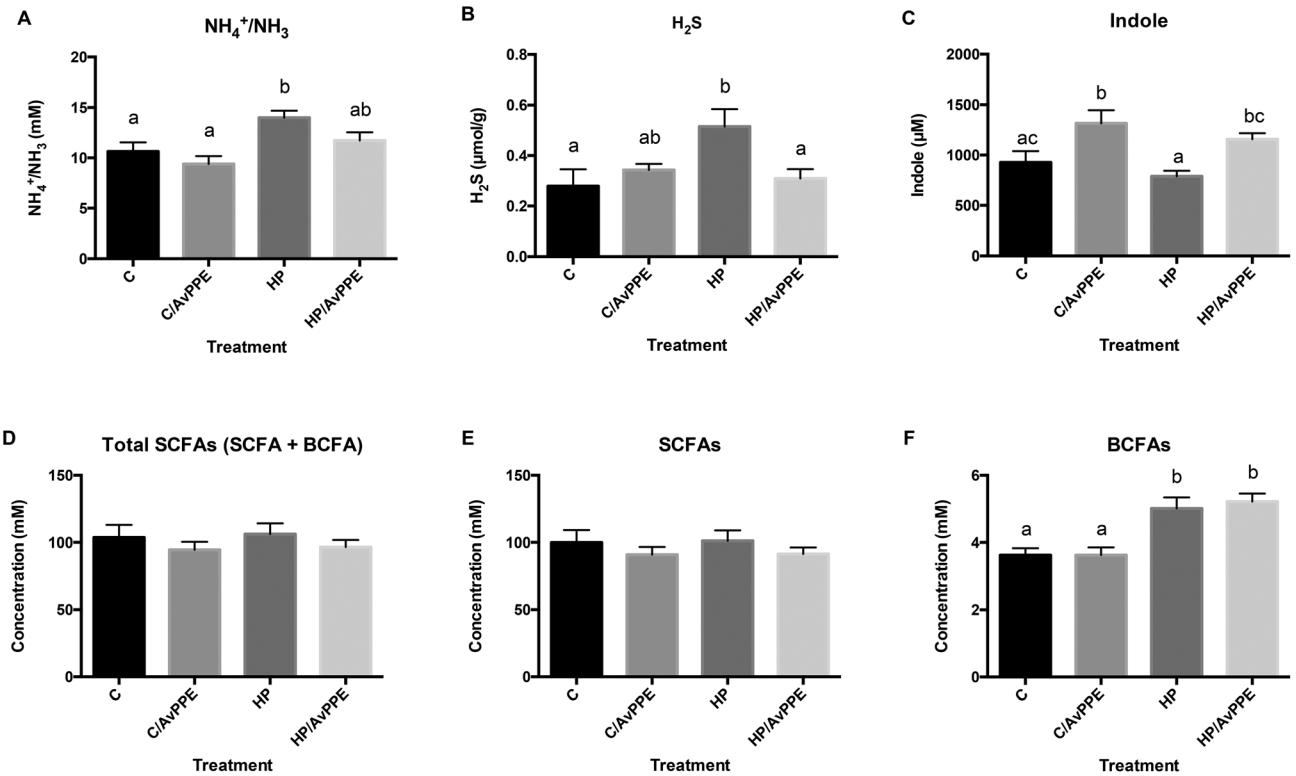

Fig. 2 Concentrations of NH_4^+/NH_3 (A), H_2S (B), indole (C), total SCFAs (SCFAs + BCFAs) (D), SCFAs (E) and BSCFAs (F) in the cecal contents. Rats were fed for 4 weeks with the different diets, with or without AvPPE supplementation. Each bar represents the average of 10 rats (with SEM). Data were analyzed by one-way ANOVA and the Tukey *post-hoc* test (p = 0.002 (A), p = 0.01 (B), p = 0.002 (C), p < 0.0001 (F)), and by the Kruskal–Wallis test and Dunn *post-hoc* test (p = 0.56 (D), p = 0.61 (E)). Bars with different letters are significantly different (p < 0.05).

The cecal concentrations of both SCFAs + BCFAs (Fig. 2D) remained unaffected by the different treatments, compared with C. When the results were analyzed separately with SCFAs and BCFAs (Fig. 2E and F, respectively), changes were only observed with the latter, which increased significantly in the HP and HP/AvPPE groups, compared with the C and C/AvPPE groups. Considering each SCFA independently, acetate and propionate were not affected by the different treatments (Fig. 3A and B) while butyrate significantly decreased under the C/AvPPE and HP/AvPPE conditions, compared with C (Fig. 3C) and valerate significantly increased under the HP/AvPPE conditions, compared with C (Fig. 3D). Both BCFAs, isobutyrate and isovalerate, significantly increased under the HP and HP/AvPPE conditions compared with C and C/AvPPE (Fig. 3E and F).

3.3 Changes in the cecal microbiota

A total of 1 763 942 sequences were obtained after trimming, assembly, quality filtering, and chimera checking, with an average of 454 pb ± 9.5 sequence length. After OTU selection, a total of 1 359 172 sequences were obtained in a BIOM table, with an average of 33 979 ± 7496 sequences per sample (range: 20 953–53 020). The rarefaction curves reached an asymptote (Fig. S1A†), indicating that the amount of sequences obtained per sample was sufficient to describe the diversity of the bacterial community of the samples, without significant differences between them. When considering the taxa with a relative abundance >0.1%, 10 phyla, 26 families and 23 genera were identified. The *core* of the microbiota, which represents the genera present in all the rats under the Control conditions, represented 37.2% of the total microorganisms (Table S1†). Neither the α diversity, evaluated through the Chao 1 index (p = 0.66), nor the β diversity evaluated from the weighted Unifrac distances (Fig. S1B†) differed between groups. The prevalence and relative abundance of the bacterial phyla, families and genera of the 4 treatments are shown in Tables S2, S3 and S4,† respectively.

Changes in the compositions of bacterial phyla, families and genera during the treatment period are shown in Fig. 4, 5 and 6, respectively. Considering globally the 4 groups of animals, the phyla Bacteroidetes and Firmicutes were the most abundant (48.2% and 38.7%, respectively), followed by Proteobacteria (6.7%) and Spirochaetes (3.1%). The other phyla, globally, had a relative abundance of 0.92%. Four phyla exhibited significant differences according to the treatment (Fig. 4). The ratio Firmicutes/Bacteroidetes was not affected by the different treatments (p = 0.06) (data not shown).

At the family level, 17.7% of the microorganisms remained unidentified, 17% was classified as Ruminococcaceae, 15.5% as S24-7, 13.3% as [Paraprevotellaceae], 11.8% as Bacteroidaceae, 4.4% as Desulfovibrionaceae, 4.2% as Veillonellaceae, 3.6% as Lachnospiraceae and 3.1% as

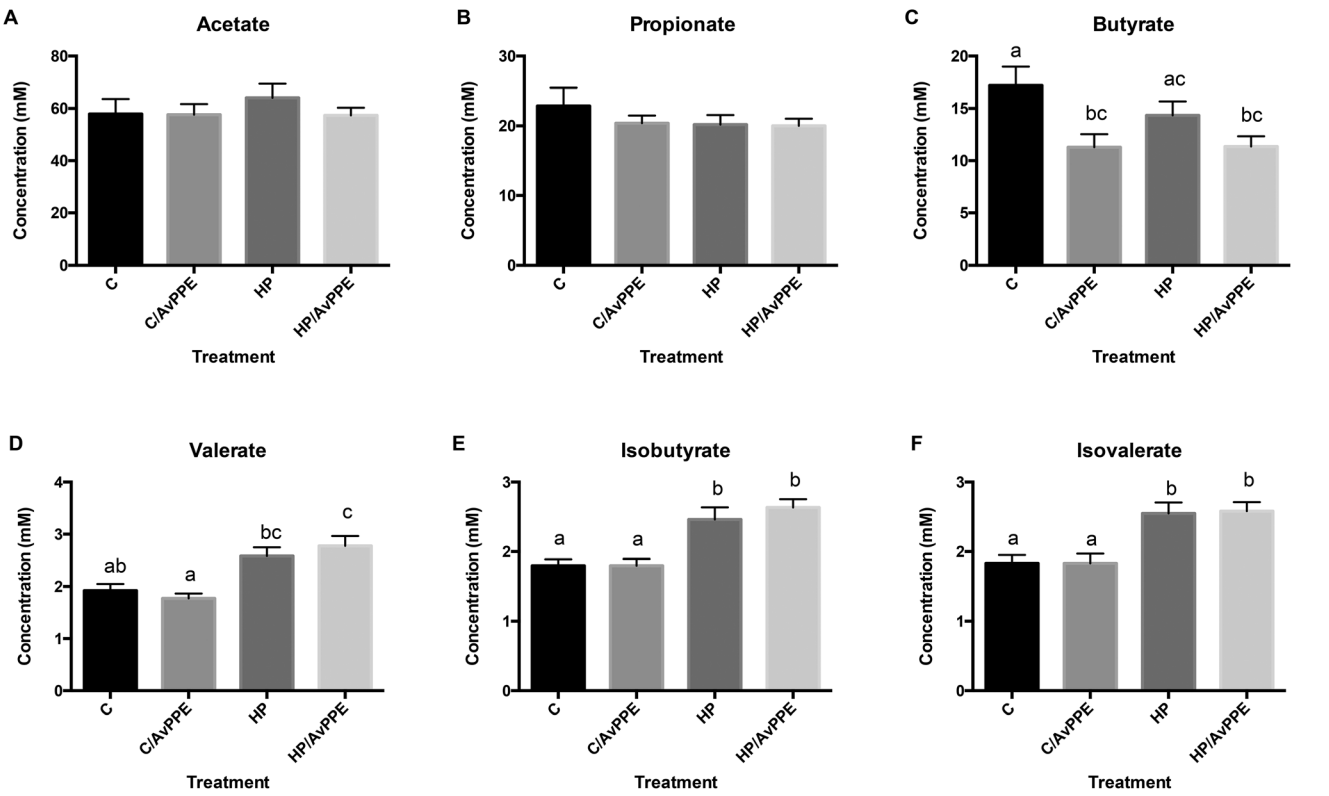

Fig. 3 Concentration of SCFAs in the cecal contents. Each bar represents the average of 10 rats (with SEM). Data were analyzed by one-way ANOVA and the Tukey *post-hoc* test (p = 0.69 (A), p = 0.57 (B), p = 0.012 (C), p < 0.0001 (E)), and by the Kruskal–Wallis and Dunn *post-hoc* test (p = 0.0002 (D), p = 0.0004 (E)). Bars with different letters are significantly different (p < 0.05).

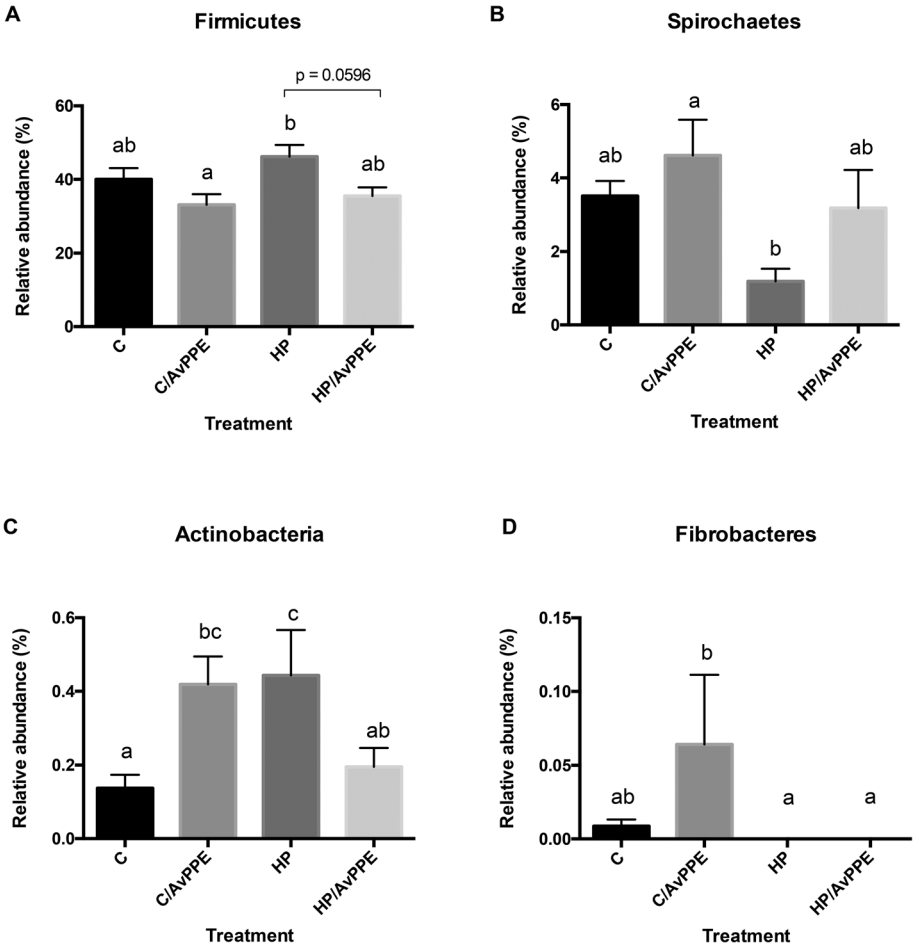

Fig. 4 Relative abundance (%) of phyla present in the cecal content which showed significant differences. Each bar represents the average of 10 rats (with SEM). Data were analyzed by one-way ANOVA (p = 0.02(A), p = 0.04(B), p = 0.04(C)), and by the Kruskal–Wallis test (p < 0.001(D)). Bars with different letters are significantly different (p < 0.05). *Post-hoc* test: Tukey (A and B), Fisher LSD (C) and Dunn (D).

Spirochaetaceae. The other families, globally, had a relative abundance of 5.2%. Seven families exhibited significant differences in their relative abundances according to the treatment applied (Fig. 5).

At the genus level, 51.2% of the microorganisms remained unidentified. *Bacteroides* was the most abundant genus (11.8%), followed by *Oscillospira* (10.1%), *[Prevotella]* (7.6%), *CF231* (5.5%), *Treponema* (3.1%), *Ruminococcus* (1.3%), *Desulfovibrio* (1.2%), *Coprococcus* (1.2%) and *Prevotella* (1%). The other genera, globally, had a relative abundance of 2.6%. Twelve genera had significant differences in their relative abundances according to the treatments (Fig. 6).

When considering the effect of the different treatments on the microbiota composition, in comparison with C, the C/AvPPE treatment only increased the phylum Actinobacteria, the families [Paraprevotellaceae] and (not significantly) the genus [*Prevotella*] (p = 0.060). Regarding the HP treatment, it increased the phylum Actinobacteria, the families Ruminococcaceae, Lactobacillaceae and (not significantly) Bacteroideceae (p = 0.078), and the genus *Lactobacillus* and (not significantly) *Bacteroides* (p = 0.078) and *Dorea* (p = 0.078). In contrast, HP decreased the genus *Prevotella* and (not significantly) the genus *Anaerovibrio* (p = 0.058). In the HP/AvPPE group, the abundances of the Actinobacteria and Firmicutes (p = 0.060) phyla, Lactobacillaceae family and *Lactobacillus* genus were lower than those in the HP group. In contrast, the abundance of the family [Paraprevotellaceae] and the genus [*Prevotella*] was higher in the HP/AvPPE group than that in the HP group. The *Prevotella*/*Bacteroides* ratio (C: 0.34, C/AvPPE: 0.13, HP: 0.02, HP/AvPPE: 0.05) was significantly lower in the HP treatment group than those in C (p = 0.0005) and C/AvPPE groups (p = 0.01).

3.4 Correlations between the different variables

Cecal ammonia correlated positively with all metabolites except indole with which the correlation was negative (Fig. S2A†). The animal body weight as well as the liver and spleen weights did not correlate with any bacterial metabolite while the kidney weight correlated positively with valerate, isovalerate and isobutyrate. Correlations between bacterial metabolites and bacterial genera are shown in Fig. S2B.† Ammonia correlated negatively with *Fibrobacter* and

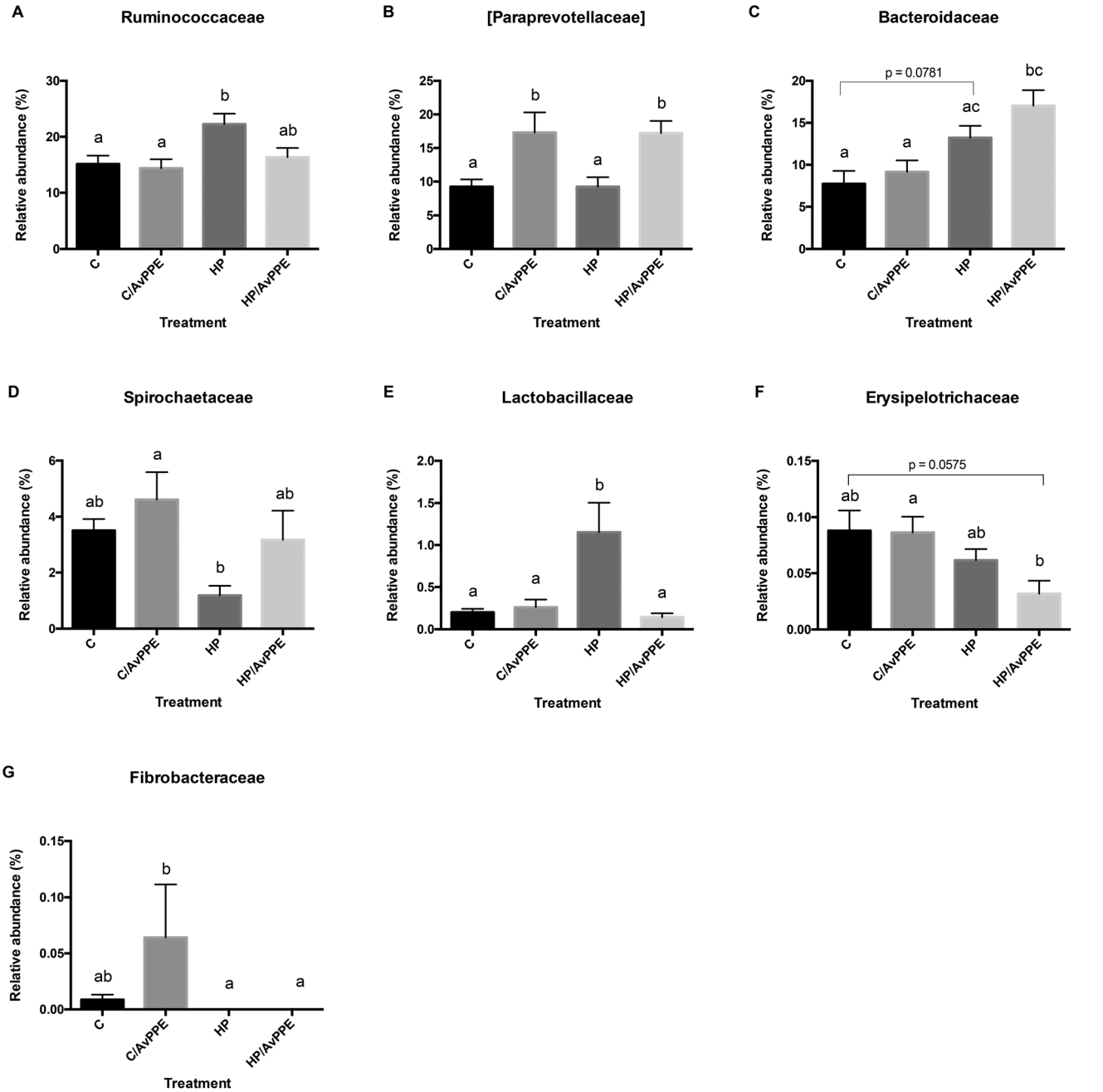

Fig. 5 Relative abundance (%) of families present in the cecal content which showed significant differences. Each bar represents the average of 10 rats (with SEM). Data were analyzed by one-way ANOVA (p = 0.01(A), p = 0.003(B), p < 0.001(C), p = 0.04(D), p = 0.00(E)) and by the Kruskal–Wallis test (p = 0.02(F), p < 0.001(G)). Bars with different letters are significantly different (p < 0.05). *Post-hoc* test: Tukey (A, B, C, D, and E) and Dunn (F and G).

Desulfovibrio and positively with *Ruminococcus*. Hydrogen sulfide did not correlate with *Desulfovibrio* but correlated negatively with *Fibrobacter*, *Anaerovibrio*, and *Roseburia* and positively with *Bacteroides*. Indole only correlated negatively with *Ruminococcus*. Regarding the SCFAs and BCFAs, most of the correlations were observed with valerate, isovalerate and isobutyrate. These BCFAs correlated positively with [*Ruminococcus*], *Bacteroides* and *Dorea* and negatively with *Anaerovibrio*, *Desulfovibrio*, *Fibrobacter* and *Bifidobacterium* (except for isovalerate). Butyrate positively correlated with *Ruminococcus*, *Sutterella* and *Treponema*.

4 Discussion

The aim of this study was to characterize the changes in the cecal concentration of amino acid-derived bacterial metabolites and the microbiota composition in rats fed a HPD and to deter-

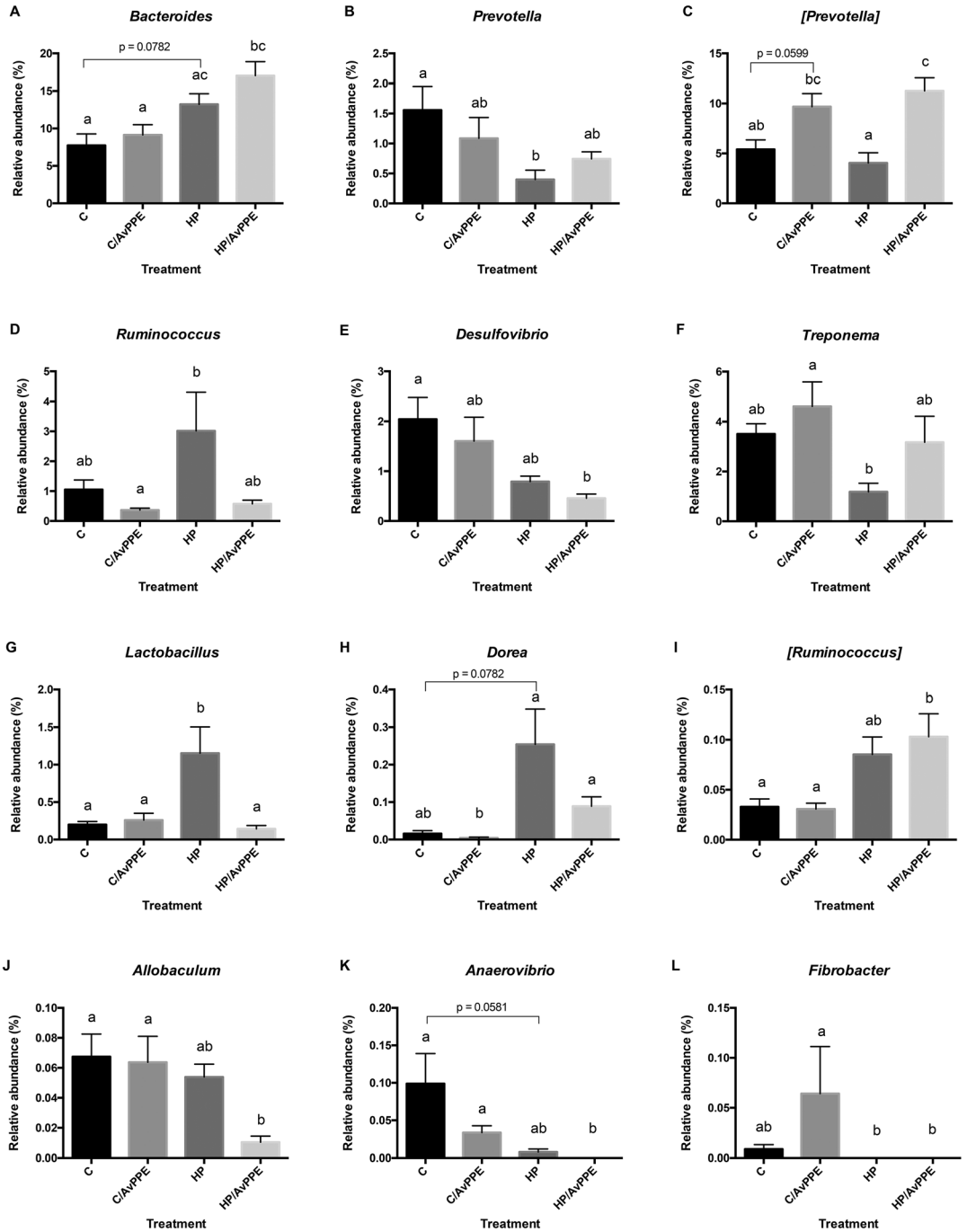

Fig. 6 Relative abundance (%) of genera present in the cecal content which showed significant differences. Each bar represents the average of 10 rats (with SEM). Data were analyzed by one-way ANOVA ($p < 0.001$(A), $p < 0.001$(C), $p = 0.04$(F), $p = 0.00$(G), $p = 0.00$(I), $p = 0.02$(J)) and by the Kruskal–Wallis test ($p = 0.05$(B), $p = 0.02$(D), $p = 0.00$(E), $p = 0.00$(H), $p = 0.00$(K), $p < 0.001$(L)). Bars with different letters are significantly different ($p < 0.05$). *Post-hoc* test: Tukey (A, C, F, G, I, and J) and Dunn (B, D, E, H, K, and L).

mine whether these changes could be prevented by the administration of a PAC-rich polyphenol extract from avocado peel.

Although HPDs are frequently used for body weight reduction,[1,2] in our study the weight gain of the animals was not affected by the HP diet, probably because the period of administration (4 weeks) was not optimal to observe any change in this variable. Indeed, a similar dietary intervention in rats showed a reduction of the body weight associated with a decreased food intake within the first days of dietary intervention.[38] However, in this latter study, the animals go back progressively to normal food consumption. In longer-term experiments, a slight decrease of the body weight was observed (10% decrease in the body weight only after 12 weeks),[39] and in humans, weight loss was observed only after 6 months.[40]

However, the short treatment period used in our study was sufficient to affect the kidney morphology, as indicated by the higher weight of these organs in the HP-fed animals. It is well known that the elimination of high amounts of plasma protein-derived metabolites generates a renal overload and enhanced glomerular pressure and hyperfiltration, resulting in enlarged renal mesangium and glomerular areas. These events are accompanied by increased kidney weight and, with time, can compromise renal function.[39,41]

We observed that the cecal concentration of ammonia was higher under the HP conditions, confirming previous results in rats and humans.[42,43] Ammonia can negatively affect the colonocytes, altering their energy supply, mitochondrial oxygen consumption, SCFA oxidation, epithelium integrity and intestinal barrier function.[5,6] AvPPE supplementation did not affect the increase of ammonia induced by the HPD, in opposition to the results from Fotschki *et al.* (2015) who reported lower levels of ammonia in the cecum of rats after 8 week-supplementation with a raspberry extract rich in ellagitannins and flavan-3-ols.[44] Such discrepancies could be explained by the fact that, in Fotschki's study, the treatment period was longer (8 weeks), the polyphenolic composition of the raspberry extract was different, with a lower PAC concentration and a high concentration of ellagitannins, absent in our extract, and the animals were fed a normo-proteic diet. Accordingly, ammonia production was probably lower than that in our study and, therefore, more easily prevented by their extract. Considering that ammonia is mainly produced by amino acid deamination and ureolysis,[45] our results suggest that the AvPPE did not inhibit the deaminase and/or urease-expressing bacterial populations.

On the other hand, our results show that the HPD increased the cecal concentrations of H_2S, confirming the observations reported by Beaumont *et al.* (2016) in rats fed a similar diet with 53% protein for 15 days.[10] A difference in this latter study is that only the total amount of H_2S was increased, and not the concentration, because of an increase of the total cecal content. H_2S in excess is toxic for the colonic epithelium by inhibiting colonocyte respiration and inducing DNA damage, and high H_2S concentrations in the colon have been associated with a higher risk of ulcerative colitis and colorectal cancer.[6,46] Accordingly, the fact that the AvPPE attenuated H_2S production may be considered as a beneficial effect. Although similar effects have been reported with grape seed and green tea PACs *in vitro*,[47,48] our study was the first evaluating this aspect in animals. Additionally, some studies have reported that dietary polyphenols bind volatile sulfur compounds in mouth[49,50] and stools,[48] and it is possible that such a mechanism explains part of our results.

Indole is a protein-derived metabolite recently described to act as a signaling molecule between the IM and the host. It increases the expression of tight-junction proteins in the epithelium, improving intestinal barrier function and attenuating inflammatory markers.[9,51] Our results show an increase in the concentrations of cecal indole in the animals from the C/AvPPE and HP/AvPPE groups, compared with the C and HP groups, an effect that might be considered as beneficial as it could contribute to the homoeostasis of the colonic epithelium. As the AvPPE does not contain indolic compounds and there is no evidence that these might derive from dietary polyphenols,[52] these results suggest that the extract selects indole-producing bacteria in the cecum such as *Prevotella* spp.,[53] which were increased in the AvPPE-treated animals and in other studies using PACs.[54]

However, it is worth noting that the positive effect of indole on the intestinal epithelium must be counterbalanced by the fact that this compound may be sulfated by phase-2 enzymes in the colonocytes or the liver, generating indoxylsulfate which, in the circulation, is considered as an uremic toxin.[55]

The cecal contents of SCFAs and BCFAs were also determined in our study. No changes in the concentrations of the total SCFAs and individual unbranched SCFAs were observed, while BCFAs increased in the animals fed the HPD, independently of AvPPE supplementation. SCFAs are bacterial fermentation products of dietary fiber and, to a lesser extent, protein, while BCFAs are exclusively derived from amino acid fermentation. Thus, the presence of BCFAs in stools or plasma represents a good marker of protein consumption.[5,6] In our study, the expected higher concentrations of isobutyrate and isovalerate in the cecum of the rats fed the HP diet were correlated positively with the abundances of *Dorea*, *Bacteroides* and [*Ruminococcus*], and negatively with those of *Anaerovibrio*, *Desulfovibrio* and *Fibrobacter*. Interestingly, we also observed that the kidney weight positively correlated with the concentrations of BCFAs. Regarding the AvPPE, it did not affect the cecal concentrations of total unbranched or branched SCFAs, but decreased cecal butyrate. This finding was also reported by Casanova-Martí *et al.* (2018) in rats supplemented for 8 days with grape seed proanthocyanidins.[56] In that latter work, the reduction in butyrate was associated with a reduction in some butyrate-producing bacteria induced by the grape seed proanthocyanidins.[56] In our work, the decrease in butyrate was not associated with a decrease in butyrate-producing bacteria induced by the AvPPE, most probably due to other events which remain to be determined.

In general, most of the protein-derived metabolites positively correlated between them except indole, maybe because this metabolite behaves differently from the others, increasing its concentration in the groups supplemented with C/AvPPE, while the other metabolites increased with the HPD.

Finally, we also evaluated the changes in the microbiota composition induced by the different treatments. Animal and human studies have reported that HPDs affect the microbiota composition, although the bacterial populations involved in these changes are highly variable according to the studies. Some of them indicate that HPDs would favor the development of a more pathogenic and pro-inflammatory microbial profile, with higher abundances of *Clostridium, Enterococcus, Shigella, E. coli* and *Bacteroides*, and lower abundances of *Bifidobacterium* and butyrate-producing bacteria including *Roseburia, E. rectale*, *C. coccoides* and *F. prausnitzii*.[21,57] However, it is globally considered that HPDs have a limited effect on the gut microbiota composition when they are not

associated with calorie restriction or a decreased fiber content.[55] Our results show that the HPD increased the phylum Actinobacteria, the families Lactobacillaceae, Ruminococcaceae and Bacteriodeaceae, and the genera *Bacteroides*, *Ruminococcus*, *Lactobacillus* and *Dorea* while it decreased *Prevotella*. The fact that both the Control and HP diets had the same type and amount of fiber and that they differed only in the protein and digestible carbohydrate contents suggests that the changes observed in the microbiota composition were only due to the higher protein content. Interestingly, a positive correlation was observed among the [*Ruminococcus*], *Dorea* and *Bacteroides* genera and the cecal concentrations of valerate, isobutyrate and isovalerate, suggesting that the increase of these taxa could explain the higher concentrations of these SCFAs in the animals fed the HPD. In contrast, a negative correlation was observed between the genera *Fibrobacter, Desulfovibrio* and *Anaerovibrio* and valerate, isobutyrate and isovalerate concentrations.

The changes in *Prevotella* and *Bacteroides* abundances observed with the HP treatment resulted in a lower *Prevotella*/*Bacteroides* ratio. The level of *Bacteroides* and *Prevotella* has been previously used to classify the human fecal communities in enterotypes.[58] The *Bacteroides* enterotype (rich in *Bacteroides* and poor in *Prevotella*) is associated with the consumption of animal protein and fat, while the *Prevotella* enterotype (rich in *Prevotella* and poor in *Bacteroides*) is more associated with the consumption of carbohydrates. Our results confirm these findings as the higher incorporation of animal protein in the diet of the animals reduced the *Prevotella*/*Bacteroides* ratio of their cecal microbiota. Similar results were also reported in dogs fed a high-protein/low-carbohydrate diet.[59]

Studies using different sources of proanthocyanidins have reported that these compounds can stimulate the growth of *C. coccoides, E. rectale, E. coli, Bifidobacterium* spp., *Lactobacillus* spp. and *Enterococcus* spp., while inhibiting that of *Clostridium* spp. and *Enterobacteriaceae*.[22–24,48] Our results indicate that the AvPPE-supplemented rats exhibited increased abundance of the Actinobacteria, [Paraprevotellaceae] and [*Prevotella*] taxa. It is therefore probable that these microorganisms are capable of metabolizing the proanthocyanidins present in the extract, releasing aromatic acids that could be beneficial for the host.

Our study presents some limitations: the dietary intervention was short, only 4 weeks, and it is possible that a longer treatment period has brought additional information. In addition, the study was centered on the microbiota and the production of bacterial metabolites, and we did not evaluate the impact of the experimental conditions (HPD and AvPPE) on rat health parameters, such as gut barrier function, and colonic or systemic inflammation. Finally, although differences in the cecal microbiota compositions among the experimental groups were detected at the end of the treatment period, it cannot be ruled out that these differences were already present before starting the treatments. However, the provenance of the sample used for the microbiota analysis (cecum) prohibited obtaining an initial sample.

In conclusion, our study confirms that the intake of HPDs increases the cecal production of ammonia and hydrogen sulfide, two microbiota-derived metabolites potentially toxic for the host. Supplementation with an avocado polyphenol extract containing a high proanthocyanidin content attenuates the production of H_2S and increases that of indole, which is considered beneficial for the gut barrier function. Such observations contribute towards explaining the health-promoting effects of proanthocyanidin-rich dietary foodstuffs including fruits and vegetables.

Abbreviations

HPDs	High-protein diets
IM	Intestinal microbiota
AvPPE	Avocado peel polyphenol extract
NP	Normal-protein
HP	High-protein
NH_4^+/NH_3	Ammonia
H_2S	Hydrogen sulfide
SCFAs	Short-chain fatty acids
BCFAs	Branched-chain fatty acids
PACs	Proanthocyanidins
C	Control
C/AVPPE	Control + AvPPE
HP/AvPPE	High-protein + AvPPE
OTUs	Operational taxonomic units
SEM.	Standard error of the mean

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Conflicts of interest

There are no conflicts of interest to declare.

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