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Metabolic consequences and gut microbiome alterations in rats consuming pork or a plant-based meat analogue

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It is unknown how human health is affected by the current increased consumption of ultra-processed plant-based meat analogues (PBMA). In the present study, rats were fed an experimental diet based on pork or a commercial PBMA, matched for protein, fat, and carbohydrate content for three weeks. Rats on the PBMA diet exhibited metabolic changes indicative of lower protein digestibility and/or dietary amino acid imbalance, alongside increased mesenteric (+38%) and retroperitoneal (+20%) fat depositions despite lower food and energy intake. In contrast, rats on the pork diet demonstrated signs of a disturbed gut–liver axis with increased liver weight (+15%) and blood low-density lipoprotein (+86%), which may have been facilitated by gut microbial changes. The colon of rats on the PBMA diet was characterized by an outgrowth of bacterial groups including Muribaculaceae, *Roseburia* and various *Eubacterium* spp. known to improve cholesterol metabolism, whereas a remarkable outgrowth of *Akkermansia*, Oscillospiraceae and Desulfovibrionaceae in rats on the pork diet may be conducive to colon mucin degradation. Effects on oxidative stress parameters were equivocal, with increased lipid oxidation (+27%) in the colon mucosa of PBMA-fed rats, whereas lower blood levels of the endogenous antioxidant glutathione (–30%) were found in pork-fed. Overall, the present rat study reveals major differences in the physiological and microbiota-related responses to diets containing either conventional pork or PBMA, which could have implications for human health.

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Introduction

There is currently a trend in high-income countries to, at least partly, replace animal protein in the diet with plant protein extracted from leguminous crops, such as pea and soybean, as well as cereal crops like wheat and rice. This trend is mainly driven by economic, societal, and ecological motivations, but also by consumer concerns about the health aspects of red and processed meat consumption.[1] These health concerns stem from epidemiological associations between high red and/or processed meat consumption and an increased risk for various chronic diseases, such as colorectal cancer, cardiovascular disease and diabetes mellitus type 2.[2–4] The underlying mechanisms explaining these associations are not yet fully clarified, but various hypotheses have been proposed, with a key role for heme-Fe. This compound, abundant in red meat, has been proposed to stimulate oxidative stress, alter the gut microbiota, and stimulate the formation of sulfides able to initiate the breakdown of the protective large intestinal mucus layer, among other mechanisms.[5,6]

In the search for meat alternatives, consumers typically do not want to compromise on food taste and texture. Food technological innovations allow the production of plant-based meat analogues (PBMA) that increasingly resemble a meat-like texture, juiciness, mouthfeel, and flavor, making these products more appealing and increasingly popular. In order to produce these types of products, various protein extraction and structuring techniques are applied, and typically involve a wide variety of ingredients and additives. Therefore, the result-

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ing food products can be considered ultra-processed foods.[7] Contrastingly, despite consumers' motivation to improve their health by replacing animal protein with alternative protein sources in their diet, a higher consumption of ultra-processed foods is associated with increased risk of developing non-communicable diseases in epidemiological studies.[8] Possible health implications related to the consumption of ultra-processed PBMA are scarcely investigated with only a few recent *in vivo* studies addressing this gap. For instance, rodent feeding trials showed that PBMA intake can influence metabolism, gut microbiota, inflammation, and colonic barrier integrity.[9–14] As van Vliet *et al.*[15] recently reviewed, novel meat alternatives may replace meat in terms of sensory experience, but not necessarily in terms of nutritional quality. Whereas meat has a balanced composition of amino acids and excellent digestibility, PBMA may contain deficiencies in certain amino acids, next to impaired digestibility due to the presence of anti-nutritional factors and/or harsh processing techniques.[16] For instance, the practice of high-moisture extrusion to provide a fibrous structure to extracted pea protein, may impair its protein digestibility.[17,18] Impaired protein digestibility may impact gut health, since a larger flow of protein could reach the colon and alter the microbial composition and activity. Certain protein fermentation markers such as phenol, *p*-cresol and ammonia can impair the gut barrier and increase gut permeability.[19] In addition, gut health may be affected by the wide variety of additives in these types of products. For instance, emulsifiers typically used in PBMA, such as carboxymethylcellulose, are increasingly suspected to contribute to obesity and the metabolic syndrome, by affecting the gut microbiota and barrier function.[20,21] In summary, the increased consumption of ultra-processed PBMA has the potential to be accompanied by undesirable health effects, which should be thoroughly investigated.

In the present research, we examined how the consumption of a popular brand of ultra-processed PBMA balls would affect gut health and metabolism in rats, compared to the consumption of model pork balls with equivalent contents of protein, fat, carbohydrates, and dry matter. A mixture of herbs was added to the model pork balls during preparation to match the type and amount of herbs used in the commercial PBMA product, after which both products were exposed to equal heating conditions. Following three weeks on the experimental diets, potential alterations in colon microbiota composition, fecal metabolites, gut histology, transcriptomics, and oxidative stress were evaluated. Additionally, we assessed systemic metabolism by NMR-based metabolomics (plasma, urine), oxidative stress, inflammation (CRP), cholesterol (LDL, HDL), lipid depositions, and organ weights.

Materials & methods

Experimental diets

Commercial PBMA balls, pork and lard were purchased from a local supermarket and meat retailer, respectively. The labeled ingredients of the commercial PBMA balls included water, pea protein, rapeseed oil, coconut oil, flavoring, smoke flavoring, rice protein, dried yeast, stabilizer (methylcellulose), potato starch, salt, potassium salt, spices, herbs in varying proportions 0.5% (parsley, rosemary, sage, basil and oregano), apple extract, garlic powder, corn vinegar, concentrated lemon juice, onion powder, pomegranate extract, emulsifier (sunflower lecithin), coloring (beetroot red), maltodextrin, and carrot powder. Pork balls were formulated to contain equal contents of protein, fat, carbohydrates, salt and herbs, as was labelled in the commercial PBMA balls. For this purpose, muscles originating from the ham of pork (76.9%) were minced with lard (16.9%), corn starch (4.7%), salt (1%) and herbs (0.5%). The herbs were a commercial mixture containing 27% dried rosemary, 27% savory, 27% oregano, and 19% thyme. The meat was minced in a grinder (Omega T-12), equipped with a 10 mm plate, followed by grinding through a 3.5 mm plate. Thereafter, the pork batch was manually shaped in balls, in equal size and dimensions as the purchased commercial PBMA balls. Pork and PBMA balls were heated in a hot air oven (Memmert) with an environmental temperature of 180 °C until a core temperature of 70 °C was reached, which was systematically monitored with a probe thermometer. Samples were heated in multiple batches and subsequently mixed within each treatment group to avoid potential batch effects. Following an overnight cool down at 4 °C, other ingredients were added to the pork and PBMA balls (Table 1) to manufacture the experimental diets. Diets were vacuum packed in daily portions (±110 g) and stored at −20 °C.

Rat feeding study

The study adhered to the principles of laboratory animal care and complied with Belgian legislation concerning animal welfare. Approval for the experimental protocol was granted by the Ethical Committee of Ghent University (ECD 22/08). The trial involved twenty male Sprague-Dawley rats (7 weeks old, approximately 200 g), obtained from Janvier Laboratories (Le Genest-Saint-Isle, France). During a five-day adaptation period, the rats were housed in groups of four under controlled environmental conditions (22.0 ± 0.6 °C, 75 ± 5% humidity, and a 15-hour light cycle) with unrestricted access to a standard laboratory diet (Ssniff R/M-N pellets, Ssniff, Soest, Germany) and water. Following the adaptation phase, the rats were housed in pairs and randomly assigned to one of two dietary treatments: a pork-based diet or a plant-based meat alternative (PBMA) diet, both provided *ad libitum* with daily refreshment. Body weight and food intake were monitored daily. On day 17, the animals were temporarily housed individually for a 24-hour period to facilitate feces collection. After 21 days of dietary intervention, the rats were anesthetized with 5% isoflurane gas, and blood was collected *via* cardiac puncture into heparinized tubes until euthanasia was complete. Plasma and red blood cells (RBC) were separated by low-speed centrifugation. Organs (brain, colon, duodenum, heart, kidney, and liver) and fat deposits (mesenteric, retroperitoneal) were excised, rinsed with 0.9% NaCl solution, and

Table 1 Ingredients and nutrients of the experimental diets

	Unit	Pork	PBMA
INGREDIENTS			
Protein product	g kg^{-1}	650	650
Commercial PBMA		—	650
Pork muscle	*g kg^{-1}*	*500*	—
Lard	*g kg^{-1}*	*110*	—
Corn starch	*g kg^{-1}*	*30.25*	—
Salt	*g kg^{-1}*	6.5	—
Herbs	*g kg^{-1}*	3.25	—
Corn starch	g kg^{-1}	160	160
Sucrose	g kg^{-1}	150	150
Cellulose	g kg^{-1}	17.8	17.8
AIN76 mineral mix (TD.79055)	g kg^{-1}	13.4	13.4
AIN76 vitamin mix	g kg^{-1}	6.3	6.3
Calcium phosphate	g kg^{-1}	1.3	1.3
Choline bitartrate	g kg^{-1}	1.2	1.2
NUTRIENTS			
Dry matter	g per 100 g	62.9	63.1
Crude protein	g per 100 g	13.8	14.2
Alanine	% protein	6.19	4.95
Arginine	% protein	6.72	8.15
Aspartic acid	% protein	10.5	11.5
Cysteine	% protein	1.19	1.35
Glutamic acid	% protein	15.5	17.4
Glycine	% protein	5.33	4.42
Histidine	% protein	4.03	2.54
Isoleucine	% protein	32.97	4.01
Leucine	% protein	8.11	8.03
Lysine	% protein	8.67	6.80
Methionine	% protein	2.36	1.16
Phenylalanine	% protein	4.11	5.33
Proline	% protein	4.47	4.73
Serine	% protein	4.69	5.86
Threonine	% protein	4.94	4.08
Tryptophan	% protein	1.28	1.10
Tyrosine	% protein	3.92	4.45
Valine	% protein	4.00	4.11
Crude fat	g per 100 g	12.0	10.7
SFA	% FAME	40.1	33.5
MUFA	% FAME	41.9	45.8
Total n-6 PUFA	% FAME	14.9	13.9
LA	% FAME	14.0	13.6
LC n-6 PUFA	% FAME	0.90	0.83
Total n-3 PUFA	% FAME	1.08	3.86
ALA	% FAME	0.87	3.81
LC n-3 PUFA	% FAME	0.23	0.18
n-6/n-3		13.8	3.59
Crude ash	g per 100 g	2.58	2.64
Oxidative status			
α-Tocopherol	mg kg^{-1}	4.4	13.3
4-HNE	μmol kg^{-1}	0.064	0.058
HEX	μmol kg^{-1}	3.19	3.63
PROP	μmol kg^{-1}	10.8	14.5
AAS	nmol mg^{-1} protein	0.319	0.583
GGS	nmol mg^{-1} protein	0.075	0.652
Pentosidine	mg kg^{-1} protein	0.036	0.388

SFA = saturated fatty acids (C08:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, C24:0), MUFA = monounsaturated fatty acids (C14:1, C16:1, C17:1, transC18:1, c9C18:1, c11C18:1, C20:1, C22:1, C24:1), ALA = α-linolenic acid, LC n-3 PUFA = omega-3 polyunsaturated fatty acids (20:3n-3; C20:4n-3; C20:5n-3; C22:5n-3; C22:6n-3), LA = linoleic acid, LC n-6 PUFA = long-chain omega-6 polyunsaturated fatty acids (20:2n-6; C20:3n-6; C20:4n-6; C22:4n-6; C22:5n-6), 4-HNE = 4-hydroxy-2-nonenal, HEX = hexanal, PROP = propanal, AAS = alpha-amino semialdehyde, GGS = gamma-glutamyl semialdehyde.

weighed. The mucosal tissue of the colon and duodenum was carefully isolated. Plasma, RBC, organ tissues, and contents of the stomach, cecum, and colon were rapidly frozen in liquid nitrogen and stored at −80 °C for subsequent analyses.

Chemical composition of diets

Diets were analyzed for dry matter (ISO 1442-1973), crude protein (ISO 937-1978), crude fat (ISO 1444-1973), and crude ash (ISO 5984-2002) content. The amino acid profile of the diets was determined by HPLC on oxidized and hydrolyzed samples, following the 2009/152/EC procedure. Lipids were extracted using chloroform/methanol (2/1; v/v), and fatty acids were methylated and analyzed by gas chromatography (HP6890, Brussels, Belgium).[22] The fatty acid profile was reported as the sum of saturated fatty acids (SFA; C08:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, C24:0), monounsaturated fatty acids (MUFA; C14:1, C16:1, C17:1, transC18:1, c9C18:1, c11C18:1, C20:1, C22:1, C24:1), polyunsaturated fatty acids (PUFA), α-linolenic acid (ALA), long-chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA; 20:3n-3; C20:4n-3; C20:5n-3; C22:5n-3; C22:6n-3), linoleic acid (LA), and long-chain omega-6 polyunsaturated fatty acids (LC n-6 PUFA; 20:2n-6; C20:3n-6; C20:4n-6; C22:4n-6; C22:5n-6). Alpha-tocopherol was measured by reverse phased HPLC (Agilent Technologies, 1200 series, Degasser, Germany).[23]

Oxidation and oxidative stress parameters

Frozen organ tissues were mixed with a phosphate buffer (50 mM, pH 7.0) at a ratio of 1 : 5 (w/v). The mixtures were homogenized using an Ultraturrax and centrifuged at 15 000*g* for 15 min at 4 °C (Avanti J-E Centrifuge, Brea, USA). The supernatant was collected and filtered through glass wool before being used for immediate analyses of thiobarbituric acid reactive substances (TBARS) and glutathione peroxidase (GSH-Px) activity. Concentrations of TBARS, representing both free and bound fractions, were measured spectrophotometrically in stomach contents, plasma, and tissue extracts using a Genesys 10S UV-VIS spectrophotometer (Thermo Scientific, Madison, USA), following the method outlined by Grotto *et al.*[24] The GSH-Px activity in plasma and organ extracts was determined by monitoring NADPH oxidation, where one unit of enzyme activity equated to the oxidation of 1 μmol of NADPH per min at 25 °C, as per Hernández *et al.*[25] Glutathione (GSH) and its oxidized form (GSSG) were quantified in RBC fractions by HPLC, with γ-glutamyl glutamate serving as the internal standard.[26] Free 4-hydroxy-2-nonenal (4-HNE), hexanal (HEX), and propanal (PROP) in diets and stomach contents were derivatized using 1,3-cyclohexanedione for quantification by HPLC-FLD, employing external standards as described by Van Hecke *et al.*[27] Levels of alpha-amino semialdehyde (AAS) and gamma-glutamyl semialdehyde (GGS) in diets and stomach contents were quantified by UPLC-FLD following derivatization with *para*-aminobenzoic acid, using an adapted protocol by Utrera *et al.*[28] Protein-bound pentosidine (PEN) in diets and plasma was quantified by HPLC-FLD

(Agilent 1200 series, Diegem, Belgium), based on the method of Scheijen *et al.*,[29] adapted by Tian *et al.*[30]

Blood biochemistry

Commercial kits for the quantification of high- (HDL), low- and very low density lipoprotein (LDL + VLDL) (MAK045), C-reactive protein (CRP; RAB0097), and lipopolysaccharide binding protein (LBP, ELK5655-96T) were purchased from Merck (Brussels, Belgium) and Gentaur (Kampenhout, Belgium). Analyses in plasma were performed in duplicate using a microplate reader (Infinite M Nano, Tecan, Grödig, Austria), according to manufacturer's instructions.

Colonic microbial composition

In brief, DNA extraction was performed by means of bead beating with a PowerLyzer (Qiagen, Venlo, the Netherlands) and phenol/chloroform extraction. Ten µL genomic DNA extract was sent out to LGC genomics GmbH (Berlin, Germany) for library preparation and sequencing on an Illumina Miseq platform with v3 chemistry with the primers 341F (5′-CCT ACG GGN GGC WGC AG-3′) and 785Rmod (5′-GAC TAC HVG GGT ATC TAA KCC-3′).[31] The total number of bacteria (bacterial density) was quantified by qPCR.[32] A table containing the relative abundance of different amplicon sequence variants (ASVs), together with their taxonomic assignments for each sample was generated using the DADA2 pipeline. The raw fastq files that were used to create the ASV table that served as a basis for the microbial community analysis, have been deposited in the National Center for Biotechnology Information (NCBI) database (accession number PRJNA1185730).

Fecal fermentation metabolites

Fecal fermentation markers were analyzed by solid-phase microextraction (carboxen-polydimethylsiloxane coated fiber, 85 µm) followed by gas chromatography-mass spectrometry (Trace DSQ II, Thermo, Finnigan), equipped with a capillary silica column (SLB-IL60, Supelco, Bellefonte, USA) (30 m × 0.25 mm × 0.2 µm).[33] Peaks were integrated for area quantification by targeting the quantification ion as follows (*m*/*z*): hexanal (44), 2-heptanone (58), acetate (60), butyrate (60), valerate (60), propionate (74), carbon disulfide (76), acetoin (88), dimethyl disulfide (94), phenol (94), *x*-cresol (108), and indole (117). Results were expressed as area under the curve (AUC) per g feces. Ammonia was measured spectrophotometrically at 625 nm in the colonic contents, with a calibration curve of NH_4Cl following the reaction with phenol, sodium nitroprusside, sodium hydroxide and sodium hypochlorite.[34]

^{1}H NMR metabolomics

Samples were thawed at 4 °C for 1 hour prior to sample preparation. Plasma samples were prepared according to a previous protocol[35] with few modifications. Briefly, thawed samples were centrifuged at 10 000*g* at 4 °C for 2 minutes before ultrafiltration. In 5 mm NMR tubes, 150 µL filtrate was mixed with 400 µL phosphate buffer (pH 7.4; 0.1 M; TMSP-d_4 = 0.8 mM). Urine samples were centrifuged at 10 000*g* at 4 °C for 5 minutes and 250 µL of the supernatant transferred to 5 mm NMR tubes and mixed with 400 µL phosphate buffer (pH 7.4; 0.3 M; TMSP-d_4 = 2.4 mM). Diets (300 mg) were extracted in duplicate by adding 1.2 mL phosphate buffer, homogenization in a commercial cell disruptor (Tissue Lyser LT) at 50 oscillations per s for 1 minute and centrifugation for 15 minutes at 10 000*g* at 4 °C. The centrifugation step was repeated in a volume of 750 µL of the supernatant. Finally, 550 µL of the extraction was added to 5 mm NMR tubes. One-dimensional (1D) high-resolution ^{1}H NMR spectra were acquired at 300 K for urine and diet samples and at 310 K for plasma samples on a Bruker Avance IVDr 600 MHz NMR spectrometer (Bruker BioSpin, Gmbh, Rheinstetten, Germany). The 1D NOESY pulse experiment with presaturation of the spectral region containing the water signal (noesygppr1d) was used with the following acquisition parameters (plasma/urine/diets): sweep width: 20 ppm, 32k data points, 128/64/64 scans, acquisition time 2.75 s. The Topspin software (Version 4.0.9) was used to process free induction decay (FID) with a line-broadening factor of 0.3 Hz, followed by Fourier transformation and automatic baseline and phase correction. Quantification of metabolites was conducted in Chenomx NMR suit 8.6 (Chenomx Inc., Alberta, Canada).

Transcriptomics

Total RNA was extracted from colonic mucosa tissues using the RNeasy Plus Universal Mini Kit (Qiagen GmbH, Hilden, Germany) in an RNase-free environment. The RNA quantity, purity, and integrity were evaluated by nanodrop, and with the Experion automated electrophoresis station, utilizing the Experion™ RNA StdSens kit (Bio-Rad, Temse, Belgium). The RNA was stored in a −80 °C freezer until shipment to NXTGNT (Ghent University, Ghent, Belgium) for massively parallel sequencing. Four samples with very low RNA integrity (RIN < 3) were excluded from sequencing, as their poor integrity persisted after repeated RNA extraction, suggesting an issue at the sampling stage. The sequencing library was constructed with the QuantSeq 3′ mRNA-Seq Library Prep Kit FWD for Illumina with the UMI Second Strand Synthesis Module. The library was sequenced as SR-75 on the AVITI Cloudbreak system (Element Biosciences). The Unique Molecular Identifiers (UMIs) were extracted and deduplicated using UMI-tools (v1.1.4) to eliminate PCR duplicates. The quality of the raw sequencing data was confirmed using FastQC (v0.12.1). FastQ Screen v0.15.3 confirmed the absence of significant contaminating DNA sequences in all samples. Using cutadapt (v4.7), sequences were processed to remove adapters and filter out low-quality reads (Phred score <20). The reads were mapped on the mRatBN7.2 rat genome using the STAR (v2.7.11) mapper. The alignment (% uniquely mapped reads) was between 75.0%–82.2% (average = 78.4%). UMI-Tools (v1.4.4) was used to deduplicate mapped reads. Feature counts at the gene and transcript isoform levels were obtained using RSEM (v1.3.0). Lowly expressed features were filtered out using the filterByExpr() function of edgeR (v4.0.16). Normalization of counts was performed with the TMM method.

Colon histology

Colonic tissue samples were processed in an automatic tissue processor under standard conditions (Shandon, Pittsburgh, PA, USA). Histological staining was performed on paraffine embedded colonic sections with periodic acid Schiff (PAS) staining to assess crypt depth and number of Goblet cells per crypt. A minimum of four sections of colon tissue were assessed, with a total of 20 reads of well-oriented crypts per animal.

Statistical analysis

Normality of distribution and homogeneity of variance of all experimental data, excluding microbial and transcriptomic datasets, were assessed using the Kolmogorov–Smirnov test and Levene's test, respectively, in SAS Enterprise Guide 7. When applicable, a mixed model ANOVA was conducted, incorporating 'protein source' as a fixed effect and 'euthanasia day' as a random effect. For datasets that failed to meet normality or homogeneity of variance assumptions, the Kruskal–Wallis Rank Sum test with pairwise comparisons (SPSS Statistics 25) was applied, with 'protein source' as the independent variable. Colon microbial composition differences across dietary treatments were determined through LEfSe (Linear Discriminant Analysis Effect Size) analysis (**https://huttenhower.sph.harvard.edu/galaxy**).[36] Taxonomic classification data used in this analysis are provided in the SI. The LEfSe analysis parameters were defined as follows: (1) alpha values for both the factorial Kruskal–Wallis test and the pairwise Wilcoxon test were set to <0.05; (2) a minimum logarithmic LDA score of 2.0 was required to identify discriminative features; and (3) the all-against-all strategy was employed for multi-class analysis. For transcriptomic analysis of colon mucosa, differential gene expression was evaluated using the edgeR package edgeR (v4.0.16) in R (v4.3.3).[37] The quasi-likelihood model was chosen to ensure robust error rate control. *P*-Values from the transcriptomic and metabolomic datasets were adjusted for multiple comparisons using the Benjamini–Hochberg procedure to mitigate false discovery rates.

Results

Diet composition

The two experimental diets had similar contents of dry matter, crude protein, crude fat, and crude ash, with notable differences observed in the composition of amino acids and fatty acids (Table 1). Compared to the pork diet, the PBMA diet exhibited 2-fold lower levels of methionine, as well as lower levels of histidine (−37%), lysine (−22%), alanine (−20%) and threonine (−17%). Conversely, the PBMA diet contained higher levels of phenylalanine (+30%), serine (+25%), arginine (+21%), tyrosine (+14%), cysteine (+13%), glutamic acid (+12%), and aspartic acid (+10%). Regarding fatty acids, the pork diet contained a relatively higher proportion of SFA compared to the PBMA diet (+20%), and markedly lower levels of ALA, which were four times higher in the PBMA diet. Other groups of fatty acids were distributed more evenly between the diets. The PBMA diet contained 3-fold higher levels of α-tocopherol, similar levels of the n-6 PUFA oxidation products 4-HNE and HEX, and 34% higher levels of the n-3 PUFA oxidation product PROP compared to the pork diet. The extent of protein (glyc)oxidation was relatively higher in the PBMA diet, as indicated by increased levels of the specific protein carbonyls AAS (+83%) and GGS (8.7-fold), and the glycoxidation marker pentosidine (10.8-fold).

Animal performance

On the first day, rats consumed approximately half as much of the PBMA diet compared to the pork diet (Fig. 1). In the following days, the difference in feed intake diminished, but overall intake remained 11% lower in rats fed the PBMA diet. Final body weight did not differ significantly across dietary treatments ($P = 0.321$). Rats consuming the PBMA diet exhibited a significantly higher mesenteric fat weight (+38%), a tendency toward increased retroperitoneal fat weight (+20%), and a significantly, yet marginally longer colon length (+4%) compared to those on the pork diet. Conversely, liver weight was significantly lower (−13%) in rats fed the PBMA diet. No significant differences were observed in the weights of the heart ($P = 0.190$) and kidneys ($P = 0.353$) between dietary treatments. The urinary pH of rats fed the PBMA diet tended to be lower compared to those on the pork diet (6.99 *vs.* 7.86, $P = 0.065$).

Blood biochemistry, oxidation & oxidative stress

The stomach contents of rats on the PBMA diet contained significantly higher levels of 4-HNE (2.8-fold), PROP (+37%), AAS (+71%) and GGS (4.2-fold) compared to the stomach contents of pork-fed rats, whereas levels of HEX ($P = 0.184$) and TBARS ($P = 0.401$) were not significantly different (Fig. 2).

Red blood cells of rats on the PBMA diet contained significantly higher levels of GSH (+43%) and GSSG (+40%) compared to rats on the pork diet, whereas its ratio was not significantly affected. Rats on the pork (*vs.* PBMA) diet had significantly higher plasma levels of VLDL + LDL (+86%), and LPS-binding protein (+16%), whereas their plasma levels of CRP (−30%) tended to be lower. Levels of TBARS ($P = 0.598$), pentosidine ($P = 0.112$) and HDL ($P = 0.377$), and activity of GSH-Px ($P = 0.146$) in plasma were not significantly affected by the dietary treatments.

Rats consuming the PBMA diet had higher levels of TBARS in the mucosa of the proximal duodenum (+10%, $P = 0.063$) and proximal colon (+27%, $P = 0.019$). The levels of TBARS in the other tissues, more specifically the liver ($P = 0.963$), kidney ($P = 0.961$) and brains ($P = 0.174$) were not affected by the dietary treatments. The activity of GSH-Px was significantly higher in the liver of rats on the pork diet, whereas the activity of this enzyme was higher in the kidney of rats on the PBMA diet. The activity of GSH-Px was not affected by the dietary treatment in duodenal mucosa ($P = 0.895$), colonic mucosa ($P = 0.176$), kidney ($P = 0.221$) and brains ($P = 0.588$).

Colon histology

No significant differences were found in the amount of Goblet cells per crypt ($P = 0.527$) or crypt depth ($P = 0.340$).

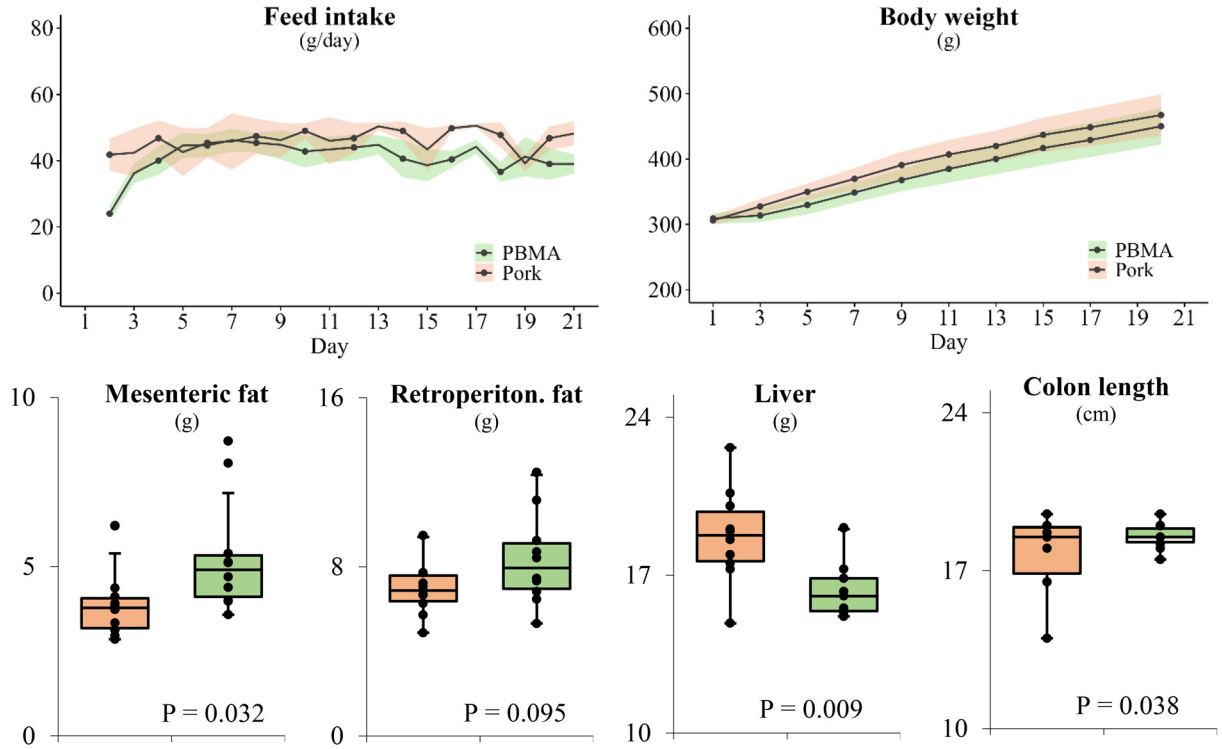

Fig. 1 Feed intake, body metrics and adiposity indicators in rats after three weeks on a pork (■) or plant-based meat analogue (PBMA) (■) diet (10 rats per treatment).

Fecal fermentation markers

The feces of rats consuming the PBMA (*vs.* pork) diet contained significantly higher levels of the protein fermentation marker phenol (4.2-fold) and a trend toward higher levels of indole (2.4-fold), accompanied by significantly higher levels of dimethyl disulfide (3.2-fold), acetone (2.4-fold), and the short chain fatty acids (SCFAs) acetate (2.7-fold), propionate (3.4-fold), butyrate (4.4-fold) and valerate (5.2-fold) (Fig. 3). Rats on the pork (*vs.* PBMA) diet contained higher levels of fecal acetoin (2.5-fold), with a tendency for higher hexanal (+76%) levels. No significant differences in carbon disulfide (P = 0.102) and 2-heptanone (P = 0.2830) were observed among treatments. Colon contents of rats on the PBMA-diet contained 21% higher levels of ammonia (P = 0.052) compared to rats on the pork-diet.

^{1}H NMR metabolomics

Sixteen urinary and 26 plasma metabolites were identified and quantified. The levels of urinary metabolites were adjusted according to their creatinine levels. Rats on the PBMA diet had significantly higher urinary levels of *cis*-aconitate (2.5-fold), and trigonelline (absent in pork), and tended to have higher urinary levels of formate (25-fold), urea (2.6-fold), hippurate (2.4-fold), and uracil (2-fold) compared to rats on the pork diet (Fig. 3). No significant differences were found in the other urinary metabolites (1-methylnicotinamide, 2-oxoglutarate, citrate, fumarate, isoleucine, leucine, threonine, trimethylamine (TMA), trimethylamine-*N*-oxide (TMAO), and valine). Plasma of rats on the PBMA (*vs.* pork) diet contained significantly higher levels of formate (2.2-fold), serine (2.0-fold), tyrosine (+69%), and ornithine (+41%), whereas significantly lower levels were found of pyruvate (−28%), alanine (−21%) and methionine (−19%). The other plasma metabolites (3-hydroxybutyrate, acetate, arginine, asparagine, citrate, glucose, glutamate, glutamine, glycine, histidine, isoleucine, lactate, leucine, lysine, phenylalanine, valine, and proline and TMAO) were not significantly different between the treatments.

Colon microbiota

The distal colon content of rats on the pork (*vs.* PBMA) diet contained a significantly higher bacterial density, and demonstrated a lower α-diversity as observed by a significantly lower Shannon index and inverted Simpson index (Fig. 4). The LEfSe analysis indicated large, significant differences in both highly abundant and lower abundant bacterial groups between rats on the experimental diets (Fig. 4, Table 2). Rats on the pork diet had higher relative abundances of *Akkermansia* (28.6 *vs.* 0.04%), Desulfovibrionaceae (6.48% *vs.* 0.91%), and Oscillospiraceae (7.94 *vs.* 3.16%), whereas they contained lower abundances of Bacteroidales (28.9 *vs.* 57.8%), mainly due to lower abundances of the Muribaculaceae (4.02% *vs.* 21.0%), *Odoribacter* (0.02 *vs.* 8.83%), and the Rikenellaceae *RC9 gut group* (0.64 *vs.* 2.24%). In addition, rats on the PBMA diet also demonstrated higher abundances of the genera *Roseburia* (3.30 *vs.* 1.39%), and *Eubacterium coprostanoligenes group* (2.33% *vs.* 0.64%), in addition to multiple other changes in generally lower abundant bacterial groups.

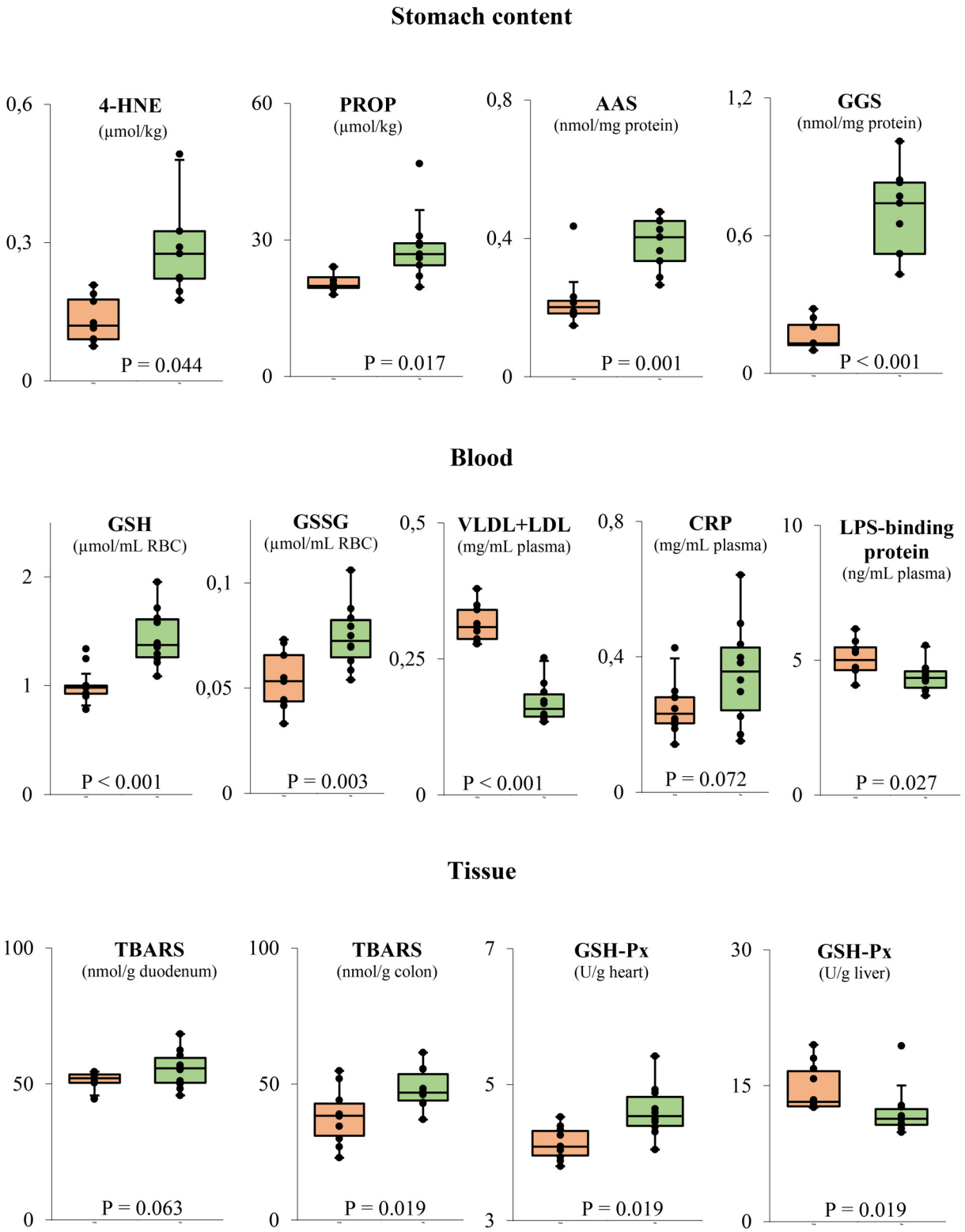

Fig. 2 Oxidation products in stomach contents, blood biochemistry, and oxidative stress parameters in tissues of rats after three weeks on a pork (▭) or plant-based meat analogue (PBMA) (▭) diet (10 rats per treatment). 4-HNE = 4-hydroxy-2-nonenal, PROP = propanal, AAS = alpha-amino semialdehyde, GGS = gamma-glutamyl semialdehyde, GSH = reduced glutathione, GSSG = oxidized glutathione, CRP = C-reactive protein, LPS = lipopolysaccharide, TBARS = thiobarbituric acid reactive substances, GSH-Px = glutathione peroxidase.

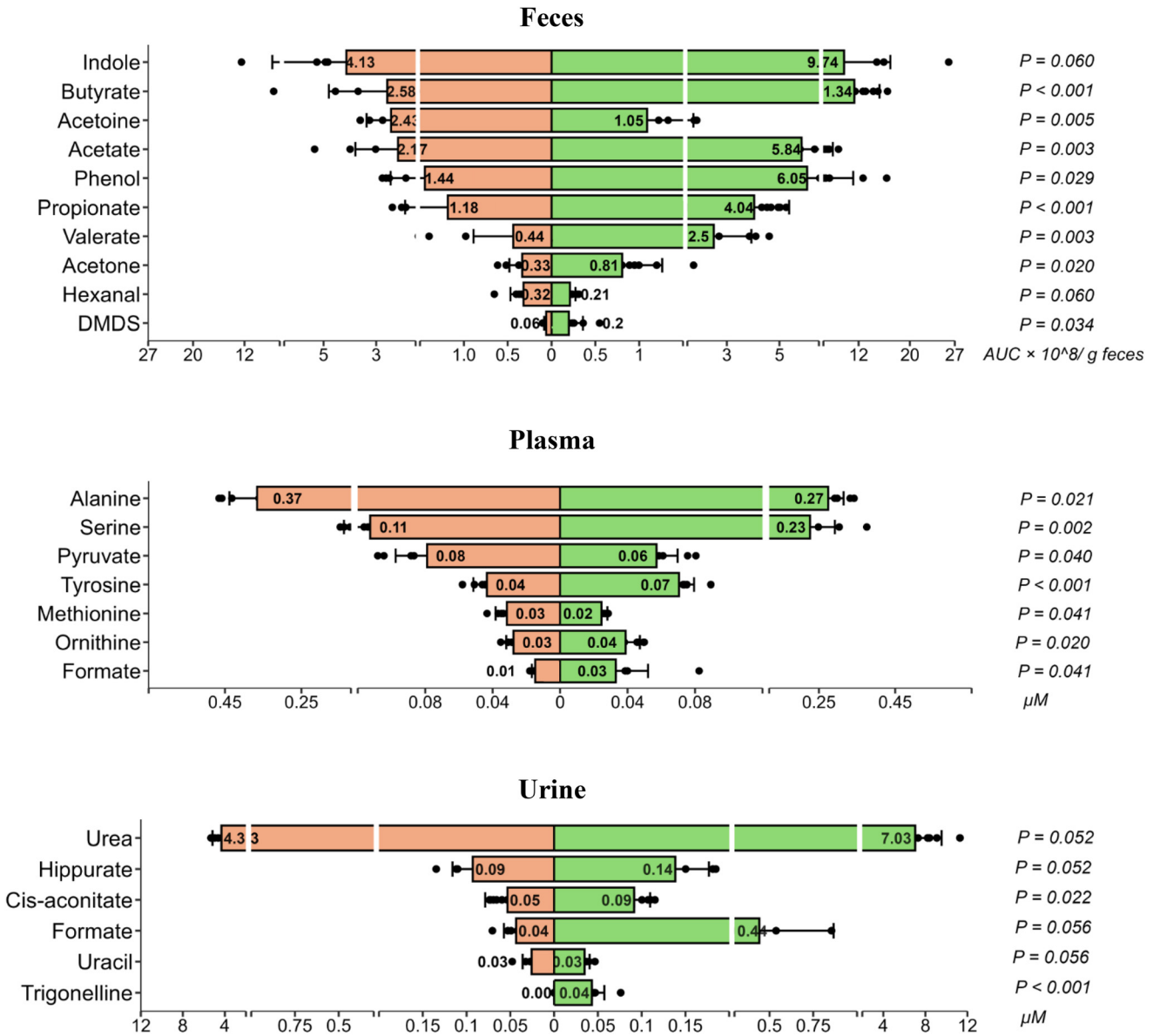

Fig. 3 Significantly altered levels of fecal, plasma and urinary metabolites in rats after three weeks on a pork (■) or plant-based meat analogue (PBMA) (■) diet (10 rats per treatment).

Transcriptomics

The count table is available as an Excel file in the SI. In our initial analysis, we identified a total of 1513 out of 12 603 identified genes that were expressed significantly different ($P < 0.05$) in response to the dietary treatment. However, after applying the Benjamini–Hochberg correction for multiple testing, none of these genes remained significant ($P_{adj} > 0.05$).

Discussion

This study showed distinctly different metabolic effects in rats following a three-week feeding period with a pork-based or a commercial PBMA-based experimental diet. Rats on the PBMA diet showed metabolic alterations associated with a lower protein digestibility and higher mesenteric and retroperitoneal fat depositions, whereas increased liver weight and blood LDL were observed in rats on the pork diet. The remarkable outgrowth of *Akkermansia*, Oscillospiraceae and Desulfovibrionaceae in rats on the pork diet suggests colon mucus degradation, although this was not directly measured here. Conversely, the colon of rats on the PBMA diet contained bacterial groups that are recognized to improve cholesterol metabolism. Effects on oxidative stress parameters were equivocal, with increased TBARS in the gut mucosa of PBMA-fed rats, and lower blood levels of the antioxidant GSH in pork-fed rats. Given both the beneficial as well as less desirable health implications, this study highlights the need for caution when

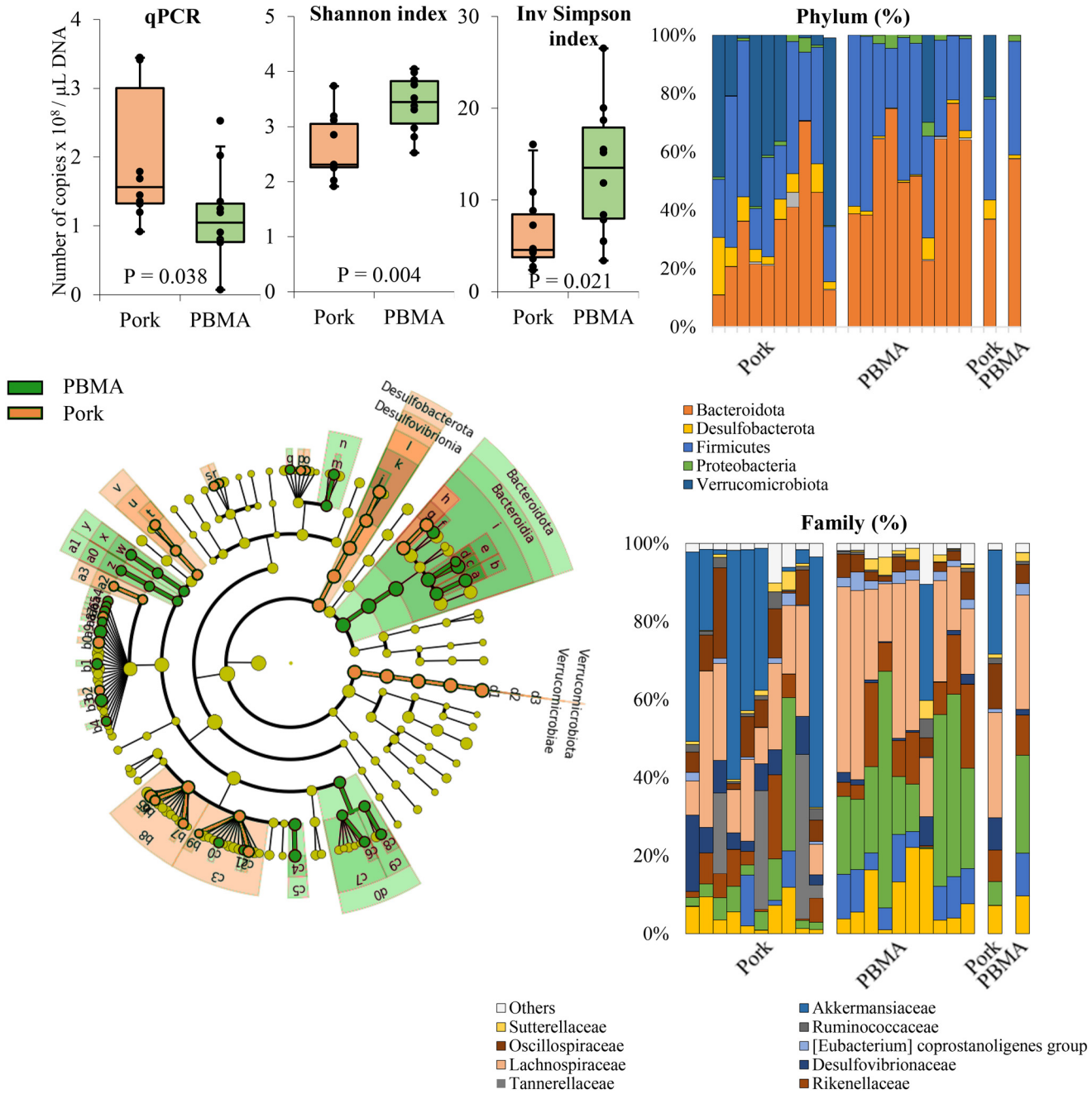

Fig. 4 Insights from qPCR, biodiversity, phylum and family-level taxonomy, and LEfSe analysis on rat colon microbiota after three weeks on a pork (▭) or plant-based meat analogue (PBMA) (▭) diet (10 rats per treatment).

transitioning from animal-derived proteins to plant-based protein alternatives.

The observed differences in the levels of various fecal, plasma, and urinary metabolites generally indicate lower protein digestibility, an unbalanced intake of amino acids and/or metabolic disturbance in rats fed the commercial PBMA diet. Specifically, the higher fecal phenol and indole levels in rats on the PBMA diet suggest increased gut fermentation of tyrosine, and tryptophan respectively. Accordingly, increased gut protein fermentation in rats on the PBMA diet was demonstrated by higher ammonia levels in the colonic contents. Taking into account that the diets were isoproteic, these results indicate a lower protein digestibility of the commercial PBMA in the proximal digestive system, leading to an increased transit of undigested protein to the colon. A similar lower protein digestibility was reported for commercial PBMAs compared to beef and pork.[10,13,38,39] The higher urinary urea levels in rats on the PBMA diet indicate increased detoxification of ammonia *via* the hepatic urea cycle. Concurrently, the accumulation of plasma ornithine, a crucial urea cycle inter-

Table 2 Heat map presenting significant differences ($P < 0.05$) among dietary treatments on the rat colonic microbiota composition using LEfSe, presented as median relative abundances (%)

	LDA	Pork	PBMA
Bacteroidota.Bacteroidia.Bacteroidales	5.17	28.9	**57.8**
Verrucomicrobiota.Verrucomicrobiae.Verrucomicrobiales.Akkermansiaceae.Akkermansia	5.09	**28.6**	0.04
Bacteroidota.Bacteroidia.Bacteroidales.**Muribaculaceae**	5.00	4.02	**21.0**
Bacteroidota.Bacteroidia.Bacteroidales.**Marinifilaceae.Odoribacter**	4.47	0.02	**8.83**
Desulfobacterota.Desulfovibrionia.Desulfovibrionales.Desulfovibrionaceae.F_Desulfovibrionaceae	4.44	**6.48**	0.91
Firmicutes.Clostridia.Lachnospirales.Lachnospiraceae.**ASF356**	4.41	0.01	**0.05**
Firmicutes.Clostridia.Oscillospirales.**Oscillospiraceae**	4.39	**7.94**	3.16
Firmicutes.Clostridia.Oscillospirales.Oscillospiraceae.**F_Oscillospiraceae**	4.32	**5.63**	1.62
Firmicutes.Clostridia.Lachnospirales.Lachnospiraceae.**F_Lachnospiraceae**	4.27	1.20	**4.00**
Bacteroidota.Bacteroidia.Bacteroidales.Rikenellaceae.**Rikenellaceae RC9 gut group**	4.09	0.64	**2.24**
Firmicutes.Clostridia.Lachnospirales.Lachnospiraceae.**Roseburia**	3.95	1.39	**3.30**
Firmicutes.Clostridia.Lachnospirales.Lachnospiraceae.**Eubacterium_xylanophilum group**	3.94	0.00	**0.07**
Firmicutes.Clostridia.Lachnospirales.Lachnospiraceae.**A2**	3.93	0.05	**0.13**
Bacteroidota.Bacteroidia.Bacteroidales.Muribaculaceae.**Muribaculum**	3.90	0.00	**0.12**
Firmicutes.Clostridia.**Peptostreptococcales_Tissierellales**	3.85	0.12	**0.65**
Firmicutes.Clostridia.Lachnospirales.Lachnospiraceae.**Acetatifactor**	3.85	0.00	**0.09**
Firmicutes.Clostridia.Oscillospirales.Ruminococcaceae.**F_Ruminococcaceae**	3.84	0.08	**0.12**
Firmicutes.Clostridia.Oscillospirales.**Eubacterium_coprostanoligenes group.F_Eubacterium_coprostanoligenes_group**	3.82	0.64	**2.33**
Firmicutes.Clostridia.**Clostridiales.Clostridiaceae.Clostridium sensu stricto1**	3.80	0.00	**0.23**
Firmicutes.Bacilli.Lactobacillales.Lactobacillaceae.**Ligilactobacillus**	3.70	**0.07**	0.00
Firmicutes.Clostridia.Oscillospirales.Oscillospiraceae.**UCG_005**	3.67	**0.08**	0.00
Firmicutes.Clostridia.Lachnospirales.**DefluviitaleaceaeUCG_011**	3.65	**0.06**	0.00
Firmicutes.Clostridia.Oscillospirales.Ruminococcaceae.**Ruminococcus**	3.64	**0.35**	0.07
Firmicutes.Clostridia.Lachnospirales.Lachnospiraceae.**GCA_900066575**	3.63	**1.64**	0.75
Firmicutes.Clostridia.Lachnospirales.Lachnospiraceae.**Lachnospiraceae FCS020 group**	3.62	0.02	**0.30**
Firmicutes.Clostridia.**ClostridiavadinBB60group.O_Clostridia vadinBB60 group**	3.60	0.00	**0.09**
Firmicutes.Clostridia.Oscillospirales.**Ruminococcaceae**	3.56	**1.07**	0.55
Firmicutes.Clostridia.Peptostreptococcales_Tissierellales.**Peptostreptococcaceae**	3.56	0.04	**0.32**
Firmicutes.Clostridia.Peptostreptococcales_Tissierellales.Peptostreptococcaceae.**Romboutsia**	3.55	0.01	**0.26**
Firm.Clostridia.Peptostreptococcales_Tissierellales.**Anaerovoracaceae**	3.51	0.04	**0.26**
Firm.Clostridia.Peptostreptococcales_Tissierellales.Anaerovoracaceae.**Eubacterium_nodatum_group**	3.48	0.02	**0.24**
Firmicutes.Clostridia.Lachnospirales.Lachnospiraceae.**Blautia**	3.35	**0.05**	0.00
Firmicutes.Clostridia.**Christensenellales.Christensenellaceae.ChristensenellaceaeR_7group**	3.34	**0.20**	0.00
Firmicutes.Bacilli.Erysipelotrichales.**Erysipelatoclostridiaceae.Erysipelatoclostridium**	3.24	**0.17**	0.00
Firmicutes.Clostridia.Oscillospirales.Oscillospiraceae.**Flavonifractor**	2.95	**0.16**	0.00

Data are ranked from high to low linear discriminant analysis (LDA) score. Bacterial groups with a median abundance <0.05% are not included. Classifications highlighted in bold represent groups with the same median values and identical LDA scores across different taxonomic levels. The LEfSe analysis conditions were as follows: (1) alpha values for the factorial Kruskal–Wallis test among classes, and for the pairwise Wilcoxon test among subclasses were less than 0.05; (2) the threshold on the logarithmic LDA score for discriminative features was set to 2.0, and (3) the strategy for multi-class analysis was all-against-all (more strict).

mediate, suggests that this detoxification mechanism was indeed enhanced in these rats. The increased ammonia detoxification on the PBMA diet can stem from higher ammonia production during protein fermentation in the gut, as observed in present study, but could originate from the breakdown of excess amino acids as well. Indeed, an unbalanced intake of amino acids can lead to the deamination of surplus amino acids, which are then metabolized through the urea cycle.[40] An imbalance in the amino acid composition of the PBMA diet is likely, due to relatively lower amounts of essential amino acids (methionine, histidine, lysine and threonine) compared to the pork diet, whilst various non-essential amino acids (serine, arginine, tyrosine, cystine, glutamic acid, and aspartic acid) were in excess.

The observed differences in blood amino acid levels may reflect the different dietary intake of these amino acids, but may potentially indicate early metabolic disturbances as well. Ebara *et al.*[41,42] found that vitamin B12 deficiency in rats led to an accumulation of serine, tyrosine, and threonine, due to decreased expression of liver enzymes responsible for their metabolism. A similar mechanism may explain the accumulation of these amino acids in rats on the PBMA diet of the present study. Indeed, the higher levels of serine (2-fold) and tyrosine (69%) in the blood of rats on the PBMA (*vs.* pork) diet were more pronounced compared to their relative differences in the diet (+25% and +14%, respectively). A clear shift in the large intestinal microbiota was observed, with rats on the commercial PBMA diet demonstrating a higher α-diversity (Shannon index, inverted Simpson index) and notable differences in the core microbiota composition. Consumption of the pork diet resulted in the highly dominant presence of *Akkermansia*, Desulfovibrionaceae spp. and Oscillospiraceae spp. in the colon, whereas the colon of rats on the PBMA diet was more dominated by Muribaculaceae, and *Odoribacter*. This is consistent with previous findings, where an outgrowth of *Akkermansia* and/or Desulfovibrionaceae was reported in rodents consuming beef (*vs.* chicken), or high levels of heme-Fe.[5,43] Additionally, *Akkermansia* and Desulfovibrionaceae have

been reported to increase in the colon of rodents on fibre-deprived diets.[44] *Akkermansia* is a specialist in the degradation of mucin, which can stimulate the renewal of the protective mucus layer in the large intestine, traditionally categorizing *Akkermansia* as a beneficial bacterium.[45] While this function is advantageous, recent studies highlight that the effects of *Akkermansia* in the gut may be context-dependent. Wolter *et al.*[46] demonstrated that a fibre-deprived diet, leading to an excessive outgrowth of *Akkermansia* in the gut, renders the host more vulnerable to mucosal pathogens. In contrast, when present in the gut of rodents on a fibre-sufficient diet, *Akkermansia* has been shown to reduce pathogen load, indicating its role in maintaining gut health under certain conditions.

The role of *Akkermansia* in the potentially excessive degradation of mucin in the present study, may allow the outgrowth of other bacteria by providing nutrients released from mucin degradation. Glycoside hydrolases from *A. muciniphila* were recently shown to remove the protective sialyl and fucosyl mucin caps, including those on double-sulfated epitopes, hereby allowing the utilization of mucin glycans by different butyrate-producing Clostridia from the Oscillospiraceae family.[47] This could explain the concomitant rise in Oscillospiraceae in the present study. Likewise, sulfate release during mucin degradation by *Akkermansia* has been proposed to support sulfate-reducing Desulfovibrionaceae,[48] which may underlie their increase in the present study. Likewise, higher levels of sulfides were observed in rodents on a diet containing beef (*vs.* chicken), higher levels of heme-Fe, and/or deprived of dietary fibre,[5,49] compounds which may affect gut health as well. For instance, low levels of H_2S may be beneficial for gut health, whereas high levels may further break down the intestinal mucus network.[50,51] In the present study, the seemingly contrasting 3.2-fold higher levels of fecal dimethyl disulfide in rats on the PBMA diet, despite their 7-fold lower relative abundances of Desulfovibrionaceae, may not be attributed to increased sulfide metabolization following mucin degradation. Since the commercial PBMA contained garlic and onion powder, which are known to be rich in various sulfur-containing compounds such as thiosulfinates, these ingredients could have contributed to the fecal dimethyl disulfide levels. However, as the dietary concentrations of these precursors were not quantified, it cannot be excluded that part of the observed dimethyl disulfide originated from microbial metabolism.

Higher relative abundances of Muribaculaceae in the colon of rats on the PBMA (*vs.* pork) diet were previously reported when replacing pork protein with pea protein,[52] the main protein source in the commercial PBMA of the present study. Interestingly, these authors suggested that Muribaculaceae were responsible for the lower blood LDL levels in rats on the pea (*vs.* pork) diet, a finding that was also observed in the present study. The influence of the gut microbiota on blood LDL could be mediated by affecting the metabolism of bile, which is formed from cholesterol in the liver.[53] Gut microbiota can interfere with the reabsorption of bile in the enterohepatic circulation, hereby promoting the fecal excretion of bile acids. In turn, the conversion of cholesterol into bile acids in the liver will be stimulated, resulting in lower plasma LDL levels. The interference of the enterohepatic circulation of bile by microbiota can occur either by directly deconjugating bile in the gut, or indirectly through bacterial produced SCFA. *Roseburia* and *Eubacterium* spp., which abundances were higher in rats on the commercial PBMA diet, are described to be a major reservoir for the enzyme bile salt hydrolase in the gut, able to deconjugate bile salts, hereby interfering in their reabsorption.[54,55] In addition, rats on the PBMA diet had higher fecal levels of all SCFA, and higher colonic abundances of various bacterial groups involved in the production of SCFA including *Odoribacter*, *Rikenellaceae RC9 gut group*, *Roseburia*, *Eubacterium_xylanophilum group* and *Eubacterium_coprostanoligenes group*. These SCFAs are absorbed into the bloodstream, and reach the liver where SCFAs can inhibit cholesterol synthesis as well.[53] All these mechanisms may contribute to the lower LDL levels observed in PBMA-fed rats, but future research should examine them more directly.

Various studies suggest that liver steatosis is ameliorated by gut Muribaculaceae,[56,57] and intestinally formed SCFA can be metabolised in the liver, influencing inflammation and lipid metabolism.[58] This may help explain the lower liver weight in rats on the PBMA diet. This proposed link between Muribaculaceae and lipid metabolism is further supported by Wang *et al.*,[12] who reported that soy-based meat analog consumption enriched gut Muribaculaceae and, compared to pork, was associated with lower serum LDL and reduced hepatic lipid accumulation in mice. In turn, liver steatosis which is usually accompanied by insulin resistance and mitochondrial dysfunction,[59] may account for the observed higher levels of pyruvate in plasma of pork-fed rats of the present study. The decreased GSH levels in red blood cells of the pork-fed rats could also be a consequence of impaired hepatic metabolism, since liver steatosis is also described to be accompanied by decreased GSH levels.[60]

In strong contrast to the effects on blood LDL and liver weight, rats on the commercial PBMA diet exhibited increased mesenteric and retroperitoneal fat deposition, hallmarks of central obesity and metabolic syndrome,[61] despite somewhat lower food consumption and energy intake. This divergence is difficult to explain and may involve mechanisms distinct from those affecting LDL and liver weight. Several hypotheses may contribute to explaining the higher mesenteric and retroperitoneal fat deposition in rats on the PBMA diet. First, visceral adiposity can be a consequence of increased gut permeability, oxidative stress and/or inflammation.[62,63] The observed higher levels of the protein fermentation markers phenol and ammonia[19] and/or present emulsifiers in the PBMA may have increased the gut permeability in rats on the PBMA diet. The use of emulsifiers, such as methylcellulose in PBMA, is increasingly suspected to contribute to obesity and the metabolic syndrome, by affecting the gut microbiota, the barrier function and inflammation.[20,21] However, the somewhat lower

plasma levels of LPS-binding protein in PBMA-fed rats do not support the hypothesis of an increased gut permeability in these rats. Also, transcriptomic analysis did not yield significant differential expression after FDR correction, including genes related to gut permeability; hence, no conclusions can be drawn from these data. Secondly, oxidative stress in duodenal and colonic mucosa, as observed by increased TBARS, and low-grade inflammation (elevated CRP) may contribute to explain the visceral adiposity in PBMA-fed rats. Several factors may have contributed to the observed increased TBARS in intestinal mucosa of PBMA-fed rats. The experimental diets contained a similar fatty acid profile with the exception of higher levels of ALA in the PBMA diet. Higher levels of TBARS in intestinal tissue may therefore be attributed to the oxidation of this n-3 PUFA. Whereas the gastric contents did not show differences in TBARS, higher levels of other lipid oxidation products (4-HNE, PROP) and protein oxidation products (AAS, GGS) were found in rats on the PBMA-diet. Contact of the intestinal mucosa with these oxidation products, may stimulate oxidative stress conditions. Increased TBARS formation was recently also demonstrated during *in vitro* gastrointestinal digestion of a vegan product, compared to beef, whereas protein oxidation measured as protein carbonyl compounds was not affected.[64] In line with this, our recent work on model pork and high-moisture extruded pea balls further showed that digested pea-based products contained similar or higher levels of lipid- and protein-(glyc)oxidation products compared to pork counterparts.[65] Lastly, oxidative stress in the gut may be initiated by the interaction with other stressors in the intestinal lumen from dietary or bacterial origins. Interestingly, a recent study also reported increased adiposity in mice consuming a high-fat commercial PBMA, with only marginal effects on liver weight compared to mice consuming a low-fat PBMA, lean pork, or beef.[14] Notably, unlike the previous study, the present study strictly controlled dietary protein, fat, and carbohydrate content, yet still observed increased visceral fat in PBMA-fed rats, underscoring that other aspects beyond macronutrients are likely involved.

In conclusion, this study revealed distinct metabolic effects in rats depending on whether they consumed a pork-based or commercial PBMA-based diet. The PBMA diet was linked with lower protein digestibility, increased gut fermentation, and higher fat deposition, while the pork diet was linked to increased liver weight and higher plasma LDL levels. Microbiota shifts, such as an outgrowth of *Akkermansia* in the pork group and higher Muribaculaceae in the PBMA group, may have contributed to these metabolic changes. Additionally, both diets affected oxidative stress parameters, though through different pathways, highlighting the complex interplay between diet composition, gut microbiota, and host metabolism. The long-term health implications of consuming PBMA remain uncertain, and further research is needed to assess the potential risks or benefits of sustained PBMA consumption over time. As this study included only male rats to avoid variability associated with the estrous cycle, future research should also consider potential sex-specific effects of PBMA consumption.

Author contributions

TVH: Conceptualization, data curation, formal analysis, funding acquisition, investigation, writing – original draft. LMAJ: Formal analysis, investigation, review & editing. XT: Formal analysis, investigation, visualization, review & editing. JVP: Formal analysis, investigation, review & editing. NEM: Formal analysis, investigation, review & editing. ASV: Formal analysis, review & editing. DD: Methodology, resources, review & editing. FVN: Methodology, resources, review & editing. GVR: Methodology, resources, review & editing. JDV: Methodology, resources, review & editing. HCB: Methodology, resources, review & editing. SDS: Conceptualization, funding acquisition, project administration, review & editing.

Conflicts of interest

No potential conflict of interest was reported by the authors.

Data availability

The raw fastq files that were used to create the ASV table that served as a basis for the microbial community analysis, have been deposited in the National Center for Biotechnology Information (NCBI) database (accession number PRJNA1185730). The count table from the transcriptomic analysis is provided as an Excel file in the SI. Supplementary information is available. See DOI: https://doi.org/10.1039/d5fo02197a.

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References

1 H. J. Lee, H. I. Yong, M. Kim, Y. S. Choi and C. Jo, Status of meat alternatives and their potential role in the future meat market—A review, *Asian-Australas. J. Anim. Sci.*, 2020, **33**(10), 1533.

2 R. Micha, S. K. Wallace and D. Mozaffarian, Red and processed meat consumption and risk of incident coronary

heart disease, stroke, and diabetes mellitus: a systematic review and meta-analysis, *Circulation*, 2010, **121**(21), 2271–2283.

3 V. Bouvard, D. Loomis, K. Z. Guyton, Y. Grosse, F. El Ghissassi, L. Benbrahim-Tallaa, N. Guha, H. Mattock and K. Straif, Carcinogenicity of consumption of red and processed meat, *Lancet Oncol.*, 2015, **16**(16), 1599–1600.

4 E. R. Hill, L. E. O'Connor, Y. Wang, C. M. Clark, B. S. McGowan, M. R. Forman and W. W. Campbell, Red and processed meat intakes and cardiovascular disease and type 2 diabetes mellitus: an umbrella systematic review and assessment of causal relations using Bradford Hill's criteria, *Crit. Rev. Food Sci. Nutr.*, 2024, **64**(9), 2423–2440.

5 N. Ijssennagger, C. Belzer, G. J. Hooiveld, J. Dekker, S. W. van Mil, M. Müller, M. Kleerebezem and R. van der Meer, Gut microbiota facilitates dietary heme-induced epithelial hyperproliferation by opening the mucus barrier in colon, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**(32), 10038–10043.

6 T. Van Hecke, J. Van Camp and S. De Smet, Oxidation during digestion of meat: interactions with the diet and helicobacter pylori gastritis, and implications on human health, *Compr. Rev. Food Sci. Food Saf.*, 2017, **16**(2), 214–233.

7 C. A. Monteiro, G. Cannon, R. B. Levy, J. C. Moubarac, M. L. Louzada, F. Rauber, N. Khandpur, G. Cediel, D. Neri and P. C. Jaime, Ultra-processed foods: what they are and how to identify them, *Public Health Nutr.*, 2019, **22**(5), 936–941.

8 G. Pagliai, M. Dinu, M. P. Madarena, M. Bonaccio, L. Iacoviello and F. Sofi, Consumption of ultra-processed foods and health status: a systematic review and meta-analysis, *Br. J. Nutr.*, 2021, **125**(3), 308–318.

9 C. Liu, J. Shi, J. Wang, Y. Dai and V. Raghavan, Effects of different processing degrees of plant-based meat on the blood biochemical level, inflammation and intestinal microorganisms in mice, *Food Res. Int.*, 2023, **173**, 113398.

10 G. Sanchez-Terrón, R. Martínez, M. J. Freire, J. Molina-Infante and M. Estévez, Gastrointestinal fate of proteins from commercial plant–based meat analogs: Silent passage through the stomach, oxidative stress in intestine, and gut dysbiosis in Wistar rats, *J. Food Sci.*, 2024, **89**(12), 10294–10316.

11 G. Sánchez-Terrón, R. Martínez, D. Morcuende and M. Estévez, Deciphering the Physiological Responses to the Intake of Plant-Based Meat Analogues: On the Track of Microbiota and Biomarkers in Serum and Urine, *J. Agric. Food Chem.*, 2025, DOI: **10.1021/acs.jafc.5c02799**.

12 H. Wang, C. Ma, Y. Li, L. Zhang, F. W. van den Berg and R. Lametsch, Dietary effects of plant-based meat analog and animal meat on serum metabolism, gut microbiota, and hepatic metabolic pathway in mice, *Food Biosci.*, 2025, **68**, 106665.

13 Y. Xie, L. Cai, Z. Huang, K. Shan, X. Xu, G. Zhou and C. Li, Plant-Based Meat Analogues Weaken Gastrointestinal Digestive Function and Show Less Digestibility Than Real Meat in Mice, *J. Agric. Food Chem.*, 2022, **70**, 12442–12455.

14 Y. Xie, L. Cai, Z. Huang, K. Shan, X. Xu, G. Zhou and C. Li, Plant-based meat analogues aggravated lipid accumulation by regulating lipid metabolism homeostasis in mice, *Food Sci. Hum. Wellness*, 2024, **13**(2), 946–960.

15 S. van Vliet, S. L. Kronberg and F. D. Provenza, Plant-based meats, human health, and climate change, *Front. Sustainable Food Syst.*, 2020, **4**, 555088.

16 S. Shaghaghian, D. J. McClements, M. Khalesi, M. Garcia-Vaquero and A. Mirzapour-Kouhdasht, Digestibility and bioavailability of plant-based proteins intended for use in meat analogues: A review, *Trends Food Sci. Technol.*, 2022, **129**, 646–656.

17 S. M. Rekola, A. Kårlund, S. Mikkonen, M. Kolehmainen, L. Pomponio and N. Sozer, Structure, texture and protein digestibility of high moisture extruded meat alternatives enriched with cereal brans, *Appl. Food Res.*, 2023, **3**(1), 100262.

18 S. West, A. J. Monteyne, G. Whelehan, D. R. Abdelrahman, A. J. Murton, T. J. Finnigan, G. Mandalari, C. Booth, P. J. Wilde, F. B. Stephens and B. T. Wall, High moisture extrusion of a dietary protein blend impairs in vitro digestion and delays in vivo postprandial plasma amino acid availability in humans, *J. Nutr.*, 2024, **154**(7), 2053–2064.

19 C. K. Yao, J. G. Muir and P. R. Gibson, Insights into colonic protein fermentation, its modulation and potential health implications, *Aliment. Pharmacol. Ther.*, 2016, **43**(2), 181–196.

20 B. Chassaing, O. Koren, J. K. Goodrich, A. C. Poole, S. Srinivasan, R. E. Ley and A. T. Gewirtz, Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome, *Nature*, 2015, **519**(7541), 92–96.

21 S. Naimi, E. Viennois, A. T. Gewirtz and B. Chassaing, Direct impact of commonly used dietary emulsifiers on human gut microbiota, *Microbiome*, 2021, **9**, 1–19.

22 K. Raes, S. De Smet and D. Demeyer, Effect of double-muscling in Belgian Blue young bulls on the intramuscular fatty acid composition with emphasis on conjugated linoleic acid and polyunsaturated fatty acids, *Anim. Sci.*, 2001, **73**(2), 253–260.

23 E. Claeys, E. Vossen and S. De Smet, Determination of α-tocopherol by reversed-phase HPLC in feed and animal-derived foods without saponification, *J. Sci. Food Agric.*, 2016, **96**(2), 522–529.

24 D. Grotto, L. D. Santa Maria, S. Boeira, J. Valentini, M. F. Charão, A. M. Moro, P. C. Nascimento, V. J. Pomblum and S. C. Garcia, Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography–visible detection, *J. Pharm. Biomed. Anal.*, 2007, **43**(2), 619–624.

25 P. Hernández, L. Zomeno, B. Ariño and A. Blasco, Antioxidant, lipolytic and proteolytic enzyme activities in pork meat from different genotypes, *Meat Sci.*, 2004, **66**(3), 525–529.

26 J. Degroote, J. Michiels, E. Claeys, A. Ovyn and S. De Smet, Changes in the pig small intestinal mucosal glutathione kinetics after weaning, *J. Anim. Sci.*, 2012, **90**, 359–361.

27 T. Van Hecke, P. L. Ho, S. Goethals and S. De Smet, The potential of herbs and spices to reduce lipid oxidation during heating and gastrointestinal digestion of a beef product, *Food Res. Int.*, 2017, **102**, 785–792.
28 M. Utrera, D. Morcuende, J. G. Rodríguez-Carpena and M. Estévez, Fluorescent HPLC for the detection of specific protein oxidation carbonyls–α-aminoadipic and γ-glutamic semialdehydes–in meat systems, *Meat Sci.*, 2011, **89**(4), 500–506.
29 J. L. Scheijen, M. P. van de Waarenburg, C. D. Stehouwer and C. G. Schalkwijk, Measurement of pentosidine in human plasma protein by a single-column high-performance liquid chromatography method with fluorescence detection, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2009, **877**(7), 610–614.
30 X. Tian, E. Vossen, S. De Smet and T. Van Hecke, Glucose addition and oven-heating of pork stimulate glycoxidation and protein carbonylation, while reducing lipid oxidation during simulated gastrointestinal digestion, *Food Chem.*, 2024, **453**, 139662.
31 A. Klindworth, E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn and F. O. Glöckner, Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies, *Nucleic Acids Res.*, 2013, **41**(1), e1.
32 J. De Vrieze, L. Raport, B. Willems, S. Verbrugge, E. Volcke, E. Meers, L. T. Angenent and N. Boon, Inoculum selection influences the biochemical methane potential of agro-industrial substrates, *Microb. Biotechnol.*, 2015, **8**, 776–786.
33 E. Vossen, S. Goethals, J. De Vrieze, N. Boon, T. Van Hecke and S. De Smet, Red and processed meat consumption within two different dietary patterns: Effect on the colon microbial community and volatile metabolites in pigs, *Food Res. Int.*, 2020, **129**, 108793.
34 A. L. Chaney and E. P. Marbach, Modified reagents for determination of urea and ammonia, *Clin. Chem.*, 1962, **8**(2), 130–132.
35 L. M. A. Jakobsen, U. K. Sundekilde, H. J. Andersen, W. Kot, J. L. C. Mejia, D. S. Nielsen, A. K. Hansen and H. C. Bertram, Administration of Bovine Milk Oligosaccharide to Weaning Gnotobiotic Mice Inoculated with a Simplified Infant Type Microbiota, *Microorganisms*, 2021, **9**(5), 1003.
36 N. Segata, J. Izard, L. Waldron, D. Gevers, L. Miropolsky, W. S. Garrett and C. Huttenhower, Metagenomic biomarker discovery and explanation, *Genome Biol.*, 2011, **12**, 1–18.
37 M. D. Robinson, D. J. McCarthy and G. K. Smyth, edgeR: a Bioconductor package for differential expression analysis of digital gene expression data, *Bioinformatics*, 2010, **26**(1), 139–140.
38 N. S. Fanelli, H. M. Bailey, T. W. Thompson, R. Delmore, M. N. Nair and H. H. Stein, Digestible indispensable amino acid score (DIAAS) is greater in animal-based burgers than in plant-based burgers if determined in pigs, *Eur. J. Nutr.*, 2022, **61**(1), 461–475.
39 Y. Xie, L. Cai, D. Zhao, H. Liu, X. Xu, G. Zhou and C. Li, Real meat and plant-based meat analogues have different in vitro protein digestibility properties, *Food Chem.*, 2022, **387**, 132917.
40 K. Tujioka, S. Lyou, Y. Fukaya, A. Sano, K. Hayase and H. Yokogoshi, Effect of adding dietary L-lysine, L-threonine and L-methionine to a low gluten diet on urea synthesis in rats, *Amino Acids*, 2005, **28**, 297–303.
41 S. Ebara, S. Toyoshima, T. Matsumura, S. Adachi, S. Takenaka, R. Yamaji, F. Watanabe, K. Miyatake, H. Inui and Y. Nakano, Cobalamin deficiency results in severe metabolic disorder of serine and threonine in rats, *Biochim. Biophys. Acta, Gen. Subj.*, 2001, **1568**(2), 111–117.
42 S. Ebara, M. Nakao, M. Tomoda, R. Yamaji, F. Watanabe, H. Inui and Y. Nakano, Vitamin B12 deficiency results in the abnormal regulation of serine dehydratase and tyrosine aminotransferase activities correlated with impairment of the adenylyl cyclase system in rat liver, *Br. J. Nutr.*, 2008, **99**(3), 503–510.
43 T. Van Hecke, J. De Vrieze, N. Boon, W. H. De Vos, E. Vossen and S. De Smet, Combined consumption of beef-based cooked mince and sucrose stimulates oxidative stress, cardiac hypertrophy, and colonic outgrowth of Desulfovibrionaceae in rats, *Mol. Nutr. Food Res.*, 2019, **63**(2), 1800962.
44 M. S. Desai, A. M. Seekatz, N. M. Koropatkin, N. Kamada, C. A. Hickey, M. Wolter, N. A. Pudlo, S. Kitamoto, N. Terrapon, A. Muller, V. B. Young, B. Henrissat, P. Wilmes, T. S. Stappenbeck, G. Núñez and E. C. Martens, A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility, *Cell*, 2016, **167**(5), 1339–1353.
45 P. D. Cani, C. Depommier, M. Derrien, A. Everard and W. M. de Vos, Akkermansia muciniphila: paradigm for next-generation beneficial microorganisms, *Nat. Rev. Gastroenterol. Hepatol.*, 2022, **19**(10), 625–637.
46 M. Wolter, E. T. Grant, M. Boudaud, N. A. Pudlo, G. V. Pereira, K. A. Eaton, E. C. Martens and M. S. Desai, Diet-driven differential response of Akkermansia muciniphila modulates pathogen susceptibility, *Mol. Syst. Biol.*, 2024, **20**(6), 596–625.
47 B. Shuoker, M. J. Pichler, C. Jin, H. Sakanaka, H. Wu, A. M. Gascueña, J. Liu, T. S. Nielsen, J. Holgersson, E. N. Karlsson, N. Juge, S. Meier, J. P. Morth, N. G. Karlsson and M. Abou Hachem, Sialidases and fucosidases of Akkermansia muciniphila are crucial for growth on mucin and nutrient sharing with mucus-associated gut bacteria, *Nat. Commun.*, 2023, **14**(1), 1833.
48 C. L. Willis, J. H. Cummings, G. Neale and G. R. Gibson, In Vitro Effects of Mucin Fermentation on the Growth of Human Colonic Sulphate-Reducing Bacteria: Ecology, *Anaerobe*, 1996, **2**(2), 117–122.
49 T. Van Hecke, E. Vossen, S. Goethals, N. Boon, J. De Vrieze and S. De Smet, In vitro and in vivo digestion of red cured cooked meat: oxidation, intestinal microbiota and fecal metabolites, *Food Res. Int.*, 2021, **142**, 110203.
50 N. Ijssennagger, R. van der Meer and S. W. van Mil, Sulfide as a mucus barrier-breaker in inflammatory bowel disease?, *Trends Mol. Med.*, 2016, **22**(3), 190–199.

51 F. Blachier, M. Beaumont and E. Kim, Cysteine-derived hydrogen sulfide and gut health: a matter of endogenous or bacterial origin, *Curr. Opin. Clin. Nutr. Metab. Care*, 2019, **22**(1), 68–75.
52 L. T. Tong, T. Xiao, L. Wang, C. Lu, L. Liu, X. Zhou, A. Wang, W. Qin and F. Wang, Plant protein reduces serum cholesterol levels in hypercholesterolemia hamsters by modulating the compositions of gut microbiota and metabolites, *iScience*, 2021, **24**(12), 103435.
53 C. Deng, J. Pan, H. Zhu and Z. Y. Chen, Effect of gut microbiota on blood cholesterol: A review on mechanisms, *Foods*, 2023, **12**(23), 4308.
54 Z. Song, Y. Cai, X. Lao, X. Wang, X. Lin, Y. Cui, P. K. Kalavagunta, J. Liao, L. Jin, J. Shang and J. Li, Taxonomic profiling and populational patterns of bac-terial bile salt hydrolase (BSH) genes based on world-wide human gut microbiome, *Microbiome*, 2019, **7**(1), 9.
55 A. Mukherjee, C. Lordan, R. P. Ross and P. D. Cotter, Gut microbes from the phylogenetically diverse genus Eubacterium and their various contributions to gut health, *Gut Microbes*, 2020, **12**(1), 1802866.
56 G. Yuan, M. Tan and X. Chen, Punicic acid ameliorates obesity and liver steatosis by regulating gut microbiota composition in mice, *Food Funct.*, 2021, **12**(17), 7897–7908.
57 J. He, Q. Le, Y. Wei, L. Yang, B. Cai, Y. Liu and B. Hong, Effect of piperine on the mitigation of obesity associated with gut microbiota alteration, *Curr. Res. Food Sci.*, 2022, **5**, 1422–1432.
58 S. Anand and S. S. Mande, Host-microbiome interactions: Gut-Liver axis and its connection with other organs, *npj Biofilms Microbiomes*, 2022, **8**(1), 89.
59 E. Scoditti, S. Sabatini, F. Carli and A. Gastaldelli, Hepatic glucose metabolism in the steatotic liver, *Nat. Rev. Gastroenterol. Hepatol.*, 2024, **21**(5), 319–334.
60 I. Grattagliano, P. Caraceni, G. Calamita, D. Ferri, I. Gargano, G. Palasciano and P. Portincasa, Severe liver steatosis correlates with nitrosative and oxidative stress in rats, *Eur. J. Clin. Invest.*, 2008, **38**(7), 523–530.
61 R. V. Shah, V. L. Murthy, S. A. Abbasi, R. Blankstein, R. Y. Kwong, A. B. Goldfine, M. Jerosch-Herold, J. A. C. Lima, J. Ding and M. A. Allison, Visceral adiposity and the risk of metabolic syndrome across body mass index: the MESA Study, *JACC Cardiovasc. Imaging*, 2014, **7**(12), 1221–1235.
62 K. M. Pou, J. M. Massaro, U. Hoffmann, R. S. Vasan, P. Maurovich-Horvat, M. G. Larson, J. F. Keaney Jr, J. B. Meigs, I. Lipinska, S. Kathiresan, J. M. Murabito, C. J. O'Donnell, E. J. Benjamin and C. S. Fox, Visceral and subcutaneous adipose tissue volumes are cross-sectionally related to markers of inflammation and oxidative stress: the Framingham Heart Study, *Circulation*, 2007, **116**(11), 1234–1241.
63 A. Gummesson, L. M. Carlsson, L. H. Storlien, F. Bäckhed, P. Lundin, L. Löfgren, Y. Y. Lam, B. Fagerberg and B. Carlsson, Intestinal permeability is associated with visceral adiposity in healthy women, *Obesity*, 2011, **19**(11), 2280–2282.
64 I. Ariz, D. Ansorena and I. Astiasaran, In vitro digestion of beef and vegan burgers cooked by microwave technology: Effects on protein and lipid fractions, *Food Res. Int.*, 2024, **186**, 114376.
65 X. Tian, J. L. Scheijen, J. Van Pee, G. Van Royen, C. G. Schalkwijk, S. De Smet and T. Van Hecke, High-moisture extruded pea balls exhibit lower (glyc)oxidative stability than pork balls following barbecuing and simulated gastrointestinal digestion, *Food Chem.*, 2025, **479**, 143864.