Urinary Metabolome Analysis Reveals Potential Microbiota Alteration and Electrophilic Burden Induced by High Red Meat Diet: Results from the French NutriNet-Santé Cohort and an in vivo Intervention Study in Rats

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List of abbreviation

CRC: colorectal cancer

CVD: cardiovascular disease

DHN-MA: dihydroxynonane mercapturic acid

HHEP-MA: 4-hydroxy-heptanol mercapturic acid

HNE-MA: 4-hydroxy-nonanal mercapturic acid

HPLA: hydroxyphenylllactic acid

HRM: high red meat

HRPC: high red and processed meat consumer

LC-MS: liquid chromatography coupled to mass spectrometry

MACs: mercapturic acid conjugates

Neu-5Gc: *N*-glycolylneuraminic acid

NRPC: non-red and processed meat consumer

OPLS-DA: orthogonal partial least square discriminant analysis

PUFA: polyunsaturated fatty acid

Keywords: Lipid peroxidation, Mercapturic acids, Metabolomics, Microbiota, Red meat

Abstract

Scope: High red and processed meat consumption is associated with several adverse outcomes such as colorectal cancer and overall global mortality. However, the underlying mechanisms remain debated and need to be elucidated.

Methods and results: Urinary untargeted LC-MS metabolomics data from 240 subjects from the French cohort NutriNet-Santé were analysed. Individuals were matched and divided into 3 groups according to their consumption of red and processed meat: high red and processed meat consumers, non-red and processed meat consumers and an at random group. Results were supported by a preclinical experiment where rats were fed either a high red meat or a control diet. Microbiota derived metabolites, in particular indoxyl sulfate and cinnamoylglycine, were found impacted by the high red meat diet in both studies, suggesting a modification of microbiota by the high red/processed meat diet. Rat microbiota sequencing analysis strengthened this observation. Although not evidenced in the human study, rat mercapturic acid profile concomitantly revealed an increased lipid peroxidation induced by high red meat diet.

Conclusion: Novel microbiota metabolites were identified as red meat consumption potential biomarkers, suggesting a deleterious effect, which could partly explain the adverse effects associated with high red and processed meat consumption.

Introduction

Over the past decades, red and processed meat intake has been associated with the development of several chronic diseases. In particular, strong associations have been reported between red and processed meat consumption and the occurrence of colorectal cancer (CRC) [1,2]. International Agency for Cancer Research (IARC) classified processed meat as carcinogenic and red meat as a probable carcinogen to humans [3]. The World Cancer Research Fund (WCRF) dose-response meta-analysis determined a 12% greater risk of developing colon cancer per 100 gram increase in red meat consumed per day and a 16% greater risk per 50 gram increase for processed meat[1]. Another recent meta-analysis reported similar conclusions [4]. Associations with cardiovascular diseases (CVD) mortality in a dose-response manner with a 16% higher risk of CVD mortality for red meat and 18% for processed meat were also reported [5].

To explain adverse health effects (notably carcinogenic) of red and processed meat excessive consumption, several hypotheses have been emitted involving various compounds among which heme iron [6,7], heterocyclic aromatic amines [8], polycyclic aromatic hydrocarbons [9], *N*-nitroso compounds [10] and *N*-glycolylneuraminic acid (Neu-5Gc) [11] appear to be the most likely ones. In particular, animal and cell model studies have emphasized the role of heme iron as a central element of the carcinogenesis induced by red and processed meat consumption [6]. Several mechanistic studies focused on the link between heme iron and CRC promotion. One of the most plausible mechanisms involves the catalytic effect of heme iron that potentializes oxidation of polyunsaturated fatty acids (PUFAs) in the colon lumen *via* reactive oxygen species production [12]. PUFAs oxidation can lead to the formation of reactive compounds such as 2-alkenals, well known as toxic compounds [13]. 2-alkenals are electrophilic compounds which, as many electrophilic species,

are mainly detoxified *via* glutathione conjugation and subsequent elimination in urine through the mercapturate pathway, as mercapturic acid conjugates (MACs) [14,15]. Considering the toxicological interest of MACs, several approaches using liquid chromatography coupled to mass spectrometry (LC-MS) have been developed to monitor the exposure to these compounds as a readout of the electrophilic burden [16,17].

Besides lipid peroxidation, it has recently been demonstrated that red meat and heme iron can induce changes in the gut microbiome. Some studies have emphasized that dietary heme iron can induce profound and non-beneficial shifts in gut microbiota composition [18,19]. A heme-enriched diet has been shown to result in the alteration of gut microbiota composition alteration, with covariations between the production of 2-alkenals and a breakdown in microbiome homeostasis in F344 rats [19]. Recent evidence suggested that gut microbiota alteration following red and processed meat consumption could be one of the contributors to CRC development [20]. In addition to these findings, Ijssennagger *et al.* underlined the possible role of gut microbiota in heme-induced hyperproliferation of colonic cells [21]. In addition to the effect of heme iron, the high protein content of red meat could also be one of the key contributors to microbiota reshaping. Several studies suggested that both the amount and the origin of dietary protein could have an impact on microbiota composition and its metabolism resulting in metabolites or co-metabolites production, i.e microbiota metabolites subsequently metabolised by the host, such as indoxyl sulphate known for its detrimental effect [22–25]. Such changes in microbiota composition and metabolism have therefore an impact on the urinary metabolome, since both have been shown to be closely linked [26].

Hence, by identifying biomarkers associated with red and processed meat consumption, this study serves a dual purpose. Firstly, the determination of biomarkers would allow a better

estimation of red meat consumption as a complement to questionnaires in cohort studies. Secondly, effect biomarker discovery, as revealed in urine, could help deciphering the mechanisms involved in the link between red/processed meat and health in future studies.

In this work, considering the ability of heme iron to both catalyse lipoperoxidation in the intestinal lumen and alter gut microbiota, we focused on the urinary metabolome as a resulting image of these deleterious effects, with a particular focus on MACs profile. We based our study on two complementary and multidisciplinary approaches: (i) urinary samples from participants of the French NutriNet-Santé cohort study and (ii) preclinical experiment conducted on rats fed either a high red meat (HRM) or a control diet (CTL), with the objective to investigate the potential impact of red and processed meat consumption on health *via* the urinary metabolome. To this end, we used LC-MS for an in-depth characterization of the urinary metabolome, based on an original approach combining a hypothesis-driven and an untargeted analysis. This approach allowed a simultaneous collection of untargeted metabolomic data for assessing microbiota alteration as revealed by urinary metabolome.

Methods

Population and collection of data and urine samples

The NutriNet-Santé study is an ongoing web-based cohort launched in 2009 in France aiming to study the associations between nutrition and health. This cohort study has been previously described in details [27]. Briefly, adults aged 18 years or older are continuously recruited among the general population and followed using a dedicated web interface (https://etude-nutrinet-sante.fr). The NutriNet-Santé study is performed in accordance with

the tenets of the Declaration of Helsinki, and all procedures have been approved by the institutional review board at the French National Institute for Health and Medical Research (IRB INSERM #0000388FWA00005831) and by the National Commission on Informatics and Liberty (CNIL #908,450 and #909,216). All participants provided informed consent and an electronic signature. The study is registered at ClinicalTrials.gov (#NCT03335644).

Upon inclusion and then every year, participants are asked to complete validated questionnaires related to sociodemographic and lifestyle characteristics, health status and medication use, dietary intakes [28–30], physical activity [31] and anthropometric characteristics [32,33]. Dietary intakes are assessed every 6 months through 3 non-consecutive, validated 24-hour dietary records, randomly distributed over 2 weeks, including 2 weekdays and 1 weekend day. Portion sizes are estimated using validated photographs, standard containers, or directly in g/L. The food content in energy, alcohol, macro- and micro-nutrients are derived from the NutriNet-Santé food composition table which is continuously updated and currently comprise >3500 items. Amounts of food consumed from composite dishes are estimated using French recipes validated by food and nutrition professionals. Dietary energy under-reporters are detected *via* the method proposed by Black [34]. Between 2011 and 2014, 19,600 volunteers participated to a clinical examination including the collection of fasting blood and urine samples that were stored at -80°C ever since.

NutriNet study sample selection

For this study, participants with at least six 24h dietary records in the 2 years preceding the urine sampling were classified according to their red and processed meat consumption (mean daily intake in g/d calculated across all 24h dietary records filled in this 2y-time-

frame). A total of 80 high consumers (HRPC) was selected among the fifth sex-specific quintile of red and processed meat consumption and matched to 80 non-consumers (NRPC) according to sex, age (5-year categories), season of urine sampling (spring-summer/fall-winter), smoking status (current smokers/non-smokers), energy intakes (quartiles) and menopausal status at urine sampling for women. Finally, 80 other participants were selected randomly among all quintiles except the non-consumers quintile and matched to the high consumer/non-consumer pairs using the same matching criteria.

Animal and diets

Four-week-old male Fischer 344 rats (10 rats/group) were purchased from Charles River. Animal experiment was authorized by the French Ministry for Higher Education, Research and Innovation (MESRI) in accordance with the local ethic committee evaluation (APAFiS #22200-2019093015339804v2). Rats were housed in conventional cages by groups of 3 or 4. Rats were randomly assigned to their cages and cages randomly placed in the animal facility. They were allowed to 10 days of acclimatisation before the start of experiment. During this period, all rats consumed the control diet. Rats had unlimited access to tap water and diet. Diets were given every day at the end of afternoon to limit oxidation during the day, as rats eat during the night. After 16 days of experimental diet, rats were placed in plastic metabolic cages. Urine was collected and food consumption was recorded after 24h of housing. Once collected, urine was stored at -80°C prior analysis. Faeces for bacterial community analysis were collected after 21 days of experiment and directly frozen in liquid nitrogen. Rats were monitored throughout the experimentation and excluded if they exhibited any of the following characteristics: weight loss > 10%, overgrown teeth or any other sign of animal suffering. No rats were excluded during this experiment. The control

diet was a modified AIN-76 diet prepared and formulated in a powdered form by SAAJ (INRAE, Jouy-en-Josas, France). All diets contained 5% safflower oil (MP Biomedicals 102888) and were low-calcium (3.5 g/kg). High red meat diet (HRM) contained 40% (on a dry weight basis) of 5% fat beef meat (Picard, France). Control diet (CTL) was adjusted for protein and fat using casein and saturated animal fat.

Sample preparation

Sample collection has already been described elsewhere [35]. Once collected samples were specifically diluted in Ultrapure water according to their osmolality measured using a freezing point osmometer (Loeser Messtechnik, Berlin, Germany). After dilution samples were centrifuged at 9500g during 5 min. The supernatant was collected and transferred into vials to be directly analysed by UHPLC-MS. QC samples were prepared either by pooling equivalent volumes of all samples or by pooling all samples from one experimental group to obtain QC group. QC of all samples were diluted accordingly to obtain dQC samples.

Dihydroxynonane mercapuric acid (DHN-MA) Immunoassay

Urinary DHN-MA concentration was determined by competitive enzyme immunoassay (EIA) as previously described [36]. Urines were assayed after dilution and normalisation in EIA buffer. Detection was recorded at 414 nm.

Bacterial community analysis

Purified genomic DNA was obtained from snap frozen faecal samples and 16S rRNA gene was amplified as described elsewhere [37]. The resulting purified amplicon libraries were loaded onto the Illumina Miseq cartridge. Valid raw sequences obtained after sequencing were processed using the FROGS pipeline [38] (Galaxy Version 3.2.3). Each pair-end valid denoised sequences were filtered, merged and clustered (swarm fastidious option using a

maximum aggregation distance of 1) [39]. After removal of putative chimera, 306 clusters satisfying the following criteria were kept: abundance representing at least 0,005 % of all sequences, presents at least 3 times in a minimum of 5% of total samples. An average of 50 810 initial valid raw reads per sample was obtained and normalized to 27512 reads per sample after rarefaction. Affiliation of Operational Taxonomic Units (OTUs) was performed using the silva 138.1_16S reference database and Blast+with equal multi-hits were manually verified and corrected if necessary. Community profiles (including α and β diversity) were determined using Phyloseq R package (v1.34.0). Chao-1 index was used to determine the within sample community richness. Divergence of microbiota composition between samples was explored using the Unifrac distance matrices and model robustness was assessed by performing permutational multivariate analysis of variance (Adonis test with 9999 permutations). OTUs were agglomerated at the genus rank to examine differential abundances in response to the type of diet using the DESeq2 R package (v1.30.1) with Wald method. Univariate tests were corrected for false discovery rate (FDR) by Benjamini-Hochberg method, and adjusted p values less than 0.05 were considered statistically significant. LDA (Linear Discriminant Analysis) effect size (LEfSe, [40]) was also estimated between the two diets (factorial Kruskall-Wallis sum-rank test) and discriminating features with $p<0.01$ and an absolute logarithmic LDA score >2.5 were represented using a cladogram.

LC-MS Analyses

All solvents were purchased from Fisher Scientific (Thermo Fisher Scientific, Illkirch, France) and were LC-MS grade. A Q-ToF Synapt G2-Si mass spectrometer operating in sensitivity mode (Waters, Manchester, UK) coupled to an ACQUITY^{TM} liquid chromatographic system

(Waters, Manchester, UK) was used to perform sample analyses according to an already described method [17]. Briefly, 10 µL of sample were injected onto a Hypersil Gold C18 (1.9 µm, 100 x 2.1 mm) analytical column (Thermo Fisher Scientific, Illkirch, France). The column was kept at 40°C and the flow rate set at 0.3 mL/min. A linear gradient elution was used with mobile phase A: 95 % H2O / 5 % methanol / 0.1 % acetic acid, and mobile phase B: 100% methanol / 0.1% acetic acid. The mobile phase composition increased from 0 to 100 % of B in 30 min, held for 4 min prior to switching back to initial conditions in 1 min and held for 5 min to re-equilibrate the system. For all injections lock-mass correction was applied by infusing leucine enkephalin 10ng.mL^{-1} at 10 uL.min. Samples were analysed in two pseudo-randomized analytical batches. dQC samples were injected at the beginning of the first batch then QC samples were regularly injected every 8 samples.

Mass spectra and Data Independent Acquisition (MS^E) spectra were acquired from *m/z* 50 to 800 using the negative ionization mode and the following set of parameters: capillary voltage 0.5 kV, sampling cone voltage 30 V, source temperature 120°C, desolvation temperature 550°C, cone gas (N_2) and desolvation gas (N_2) 30 and 600 L/H, respectively. The trap collision energy was set at 15eV for MS^E acquisitions.

Data Processing

Mass spectral data processing was achieved using XCMS 3.0 with the Workflow4Metabolomics (W4M) collaborative environment dedicated to the processing of metabolomics data [41]. Centwave algorithm was used to perform automatic data extraction using the following set of parameters: ppm= 10, peakwidth = (5,40), sntresh = 10, noise = 10000, bw =10, mzwid = 0.01. Data filtering process was carried out using several filters: first, all features displaying a correlation coefficient with dQC samples lower than 0.6 were

eliminated. Then, features whose ratio of the average signal in QCs over average signal in blanks was lower than 3 were eliminated. Finally, after performing LOESS correction to avoid batch effects, features displaying RSD values lower than 30% in QC samples were kept for further processing.

Data from MS^E experiments performed on group-QC samples were processed according to Jamin *et al.* [17] using the UNIFI software (Waters, Manchester, UK). Only features displaying the characteristic neutral loss of mercapturic acids (129.042) in every replicate (n=6) within a group QC-sample were considered. Then targeted MS/MS fragmentation experiments at 15eV using an isolation parameter "LM resolution" set at 15 a.u, were carried out to confirm the detected neutral loss.

Metabolite Identification

Features annotation was performed by matching in-house database to the obtained peak list. For every putative annotation, the experimental MS/MS spectrum was compared to our in-house database of standard metabolites. Features displaying similar *m/z* (within a 5ppm window), retention time (within a 0.5min + 0.016*RT window) and fragmentation pattern were considered as level 1 identification according to Sumner et al [42].

Statistical analyses

Statistical analyses were achieved using Simca-P 14.0 software (Umetrics, Umea, Sweden). All data were log transformed and Pareto scaled prior orthogonal partial least square discriminant analysis (OPLS-DA). Predictive capacity (Q^2) and permutation test were used to check validity and robustness of OPLS-DA models. OPLS-DA model was considered valid if displayed Q2 intercept < 0.05 and Q^2cum >0.4 [43].

However, since OPLS-DA does not allow to adjust for confounding factors in the model and

cannot account for the matching of individuals, penalized conditional logistic regression models were used to determine which metabolites were most associated to red and processed meat consumption as well as the direction of the associations (using Odds Ratios), while accounting for the matched design and adjusting for potential confounders. ElasticNet penalization [44] available in the R penalizedclr package was used to allow variable selection by removing the less predictive variables. The first model included all annotated metabolites, identified MACs and: physical activity, BMI, level of education, alcohol consumption and energy intake. Once intensities of ions were scaled to unit-variance, the ElasticNet parameters α and λ were optimized using 7-fold cross validation repeated 100 times. α refers to the partition coefficient between RIDGE and LASSO regression (α=1 correspond to LASSO only) and λ corresponds to the strength of penalization. Optimal values were determined as α=0.3625 and λ=2.44. The model was calculated with optimized parameters and its stability was evaluated by bootstrap resampling by selecting 80% of dataset and repeated 1000 times. Only variables selected in the initial model and over 40% of bootstrap iteration were considered according to Lécuyer et al[45]. Dietary intakes of fibres and calcium were additionally included in the models using the same α and λ parameters to investigate the potential influence of these variables in the red and processed meat high- and non-consumer discrimination. Further adjustments were tested by considering other nutritional parameters such as polyphenol, vitamin, fruits and heme iron intake. All tested combinations are displayed in the Supplementary Table1.

Results

Table 1 describes the studied population socio-demographic, lifestyle and dietary characteristics for the three groups of subjects: high red/processed meat consumers, non-consumers and randomly selected participants.

To investigate the potential deleterious effects of high red and processed meat consumption, untargeted metabolomics analysis was performed on urine with an LC-MS approach aiming at a dual purpose. In first instance, urinary metabolome was considered as a whole to investigate the global impact of red and processed meat consumption with respect to the three groups selected from the NutriNet-Santé study. In parallel, considering the toxicological interest of MACs as markers of exposure to reactive substances, special attention was paid to these compounds *via* a specific screening workflow enabling the extraction of mass spectrometric signals coming from MACs (Jamin *et al.*, 2020).

The LC-MS metabolomic analysis led to the extraction of 8834 MS features, yielding 4138 features after filtration. Among these features , 63 metabolites were annotated at level 1 according to Metabolomics Standard Initiative classification [42] (see Supplementary Table 2) and 30 metabolites were putatively identified as MACs on the basis of their characteristic neutral loss of the *N*-acetyl cysteine moiety (129.042 a.m.u.) confirmed by targeted MS/MS experiments (see Supplementary Table 3). However, among these 30 MACs, only a few could be formally annotated. In first instance, an OPLS-DA analysis was performed using the whole dataset (i.e. the 4138 features). A valid model (Q^2= 0.409, intercept = -0.291) could be constructed for discriminating HRPC and NRPC. According to this model, 1189 features displayed a ViP score > 1. The related score plot and the permutation tests are available in

Supplementary Figure 1. This result suggested that many urinary metabolites are affected by the diet and likely associated with red and processed meat consumption.

In order to consider potential confounding factors and the matching of samples, a penalized logistic regression analysis was performed based on the selection of all the 63 annotated metabolites and the 30 confirmed MACs. First, only non-nutritional parameters were used as covariates to account for differences other than dietary habits between HRPC and NRPC. Therefore, the first model was adjusted on BMI, physical activity, level of education, alcohol consumption and caloric intake.

Among the 93 ions constituting the dataset, 8 were found negatively associated to red and processed meat consumption and 1 was found positively associated, as shown in **Figures 1A and 1B**. When compared to the NRPC, HRPC exhibited lower level of: cinnammoylglycine (OR= 0.54 , 95% CI = [0.33-0.84]), methoxysalicylic acid, (0.31, [0.13-0.65]), 2,2-dimethylglutaric acid (0.55, [0.33-0.87]), *N*-acetyl-leucine (0.58, [0.34-0.92]), *p*-coumaric acid (0.51, [0.28-0.85]), hydroxyphenyllactic acid (0.55, [0.3-0.93]), 10-hydroxydecanoic acid (0.3, [0.11-0.69]) and finally one unknown mercapturate , named as Merca14 (0.47, [0.28-0.74]). Interestingly, only one annotated metabolite and no mercapturate was found positively associated to a higher intake of red and processed meat. The only metabolite found to be positively associated with high intake of red and processed meat was indoxyl sulphate (1.59, [1.06-2.47]). Correlations between red and processed meat intake for all 240 participants and metabolite levels are shown in Figure 1B. The strongest negative correlation was observed for cinnamoylglycine and the strongest positive correlation for indoxyl sulphate.

To further investigate the influence of other nutritional parameters and because of their

significantly lower intake in high red and processed meat consumers and their association with health, notably CRC risk [46], dietary intakes of fibres and calcium were additionally included in the models.

When the model was further adjusted for calcium intake, only a few changes occurred: indoxyl sulphate was no longer kept in over 40% of bootstrap iteration and two mercapturates, respectively named as Merca16 (0.48, [0.27-0.77]) and Merca53 (0.56, [0.3-0.91]), were negatively associated with red and processed meat consumption. All other metabolites from the previous model were retained in the calcium adjusted model. Odd-ratio values for all models are available in Supplementary Table 1.

Interestingly, when adjusting the initial model for dietary fibre intake, the resulting metabolic signature was drastically different. Only three compounds, namely *N*-acetyl-leucine (0.47, [0.25-0.82]), tyrosine (0.58, [0.33-0.96]) and one mercapturate named as Merca15 (0.42, [0.22-0.77]), were found negatively associated with red and processed meat intake and none was found positively associated.

Finally, no valid model could be generated when adjusting the initial model for heme iron intake due to a high co-linearity between the consumption of red and processed meat and the heme iron intake.

Concerning the preclinical experiment, a valid OPLS-DA model (Q^2= 0.899, intercept = -0.18), displayed in Supplementary Figure 2, was generated to discriminate high red meat (HRM) and control (CTL) diets. From MS data 3470 features were kept after data filtration and 920 exhibited a ViP score > 1. Among the nine metabolites found significantly different between groups in the human cohort, five were also annotated in the animal experiment: cinnamoylglycine, indoxyl sulphate, *N*-acetyl-leucine, *p*-coumaric acid and 2,2-

dimethylglutaric acid. Three of them were significantly altered between the two experimental groups as displayed in Figure 1C to E: cinnamoylglycine (fold change= 0.19, *p*-value = < 0.0001, unpaired t-test), indoxyl sulphate (1.7, 0.043) and *p*-coumaric acid (3.39, 0.0014).

Among the 3470 features, 47 were formally identified as MACs and 16 were selected in the OPLS-DA model. As shown in **Figure 2**, an overall increase of the MACs amount was observed between the HRM-fed and the CTL groups. In addition, three of the sixteen significant MACs were formally annotated as dihydroxynonane mercapturic acid (DHN-MA), 4-hydroxy-nonanal mercapturic acid (HNE-MA) and 4-hydroxy-heptanol mercapturic acid (HHEP-MA). However, in the human study no change in the MACs profile was observed, especially for lipid peroxidation-derived MACs. This result was reinforced by quantification of DHN-MA, a well-known mercapturic acid linked to lipid peroxidation, which exhibited no significant difference (p-value: 0.1137) between HRPC and NRPC.

Considering the microbial origin of some of the discriminant metabolites, fecal microbiota analysis was performed by sequencing V3-V4 region of the 16S rRNA gene on samples originating from the preclinical experiment. This analysis revealed a significant impact of the diet on the microbiota composition. Although the Chao-1 index used to assess the bacterial richness was not affected by the diet (**Figure 3A**), the ordination based on UniFrac distances between samples demonstrated the occurrence of distinct profiles of bacterial community between high red meat and control diet (p-value: <0.001) as displayed in **Figure 3B**. Regarding the microbiota composition at the phylum level, significant changes were observed, namely for *Firmicutes*, *Actinobacteria*, *Bacteroidota*, *Proteobacteria* and

Deferribacterota as shown in **Figure 3C**. The first two were increased with high red meat diet whereas the other three were decreased. Within each of them, significant changes at the genus level were observed (Figure 3D), including notably significant decreases of *Bifidobacterium* (fold change= -3.66, *p*-adj = $3.17e^{-8}$) and *Parasutterella* (fold change= -1.5, *p*-adj = 0.001), and increases of *Limosilactobacillus* (fold change= 2.33, *p*-adj = $9.89e^{-8}$) and an unclassified genus belonging to *Eggerthellaceae* (fold change= 2.98, *p*-adj = $2.28e^{-11}$) with high red meat diet (**Figures 3C-D**).

Discussion

In this work, the impact of high red and processed meat consumption on the urinary metabolome has been investigated in both a human cohort and a controlled preclinical experiment on rats. In humans, modulation of this impact could be highlighted by considering other nutritional intakes differing between HRPC and NRPC, including *e.g.* calcium or fibres. MACs profile, which notably reflects the electrophilic burden of the diet, was slightly altered in the human study conversely to the preclinical experiment in which a tremendous increase of mercapturic species in urine of HRM-fed rats was observed.

In the human cohort, the urinary content of 8 metabolites and one unknown mercapturate were found modified in the initially adjusted model. This model reflects differences in eating habits between HRPC and NRPC, which go beyond the consumption of red and processed meat alone. Interestingly, several of these 8 metabolites, including cinnamoylglycine, hydroxyphenylllactic acid (HPLA) and indoxyl sulphate, are reported to have a microbial origin [47,48]. Although microbiota alteration by dietary habits is not surprising, the modulation of microbiota-derived compounds observed in this work is of particular interest since some of these metabolites had never been associated to red and processed meat

consumption. Cinnamoylglycine, which abundance is lowered in HRPC, was proposed as a marker of a healthy microbiome, as the level of this metabolite appears to be correlated with a higher diversity [49,50]. Beyond its use as a marker of diversity, this metabolite has been proposed as a marker of resistance against the colonization by *Clostridium difficile* that is known to exhibit pathogenic effects [51]. This lower level of cinnamoyglycine in HRPC could be associated to a lower microbiota diversity that could render more susceptible to colonization by pathogenic species [51]. In the same way, HPLA was found to be excreted by a highly used probiotic strain of *Lactobacillus fermentum* [52]. *In vitro,* strains belonging to *Bifidobacterium spp.* and *Lactobacillus spp.* were found to produce high amounts of HPLA that has the ability to act as an antioxidant by scavenging ROS in mitochondria [53]. On the contrary, urinary indoxyl sulphate levels were increased in HRPC subjects. Indoxyl sulphate is a well-studied uremic toxin which microbial origin has been demonstrated by comparing germ-free and conventional mice [48]. Indoxyl sulphate is originating from bacteria expressing tryptophanase that converts tryptophan into indole, which is then sulphated in the liver [47]. Therefore, this end-metabolite can be used as a biomarker of indole production by the gut microbiota [54]. Modulations of indoxyl sulphate by diet have already been described when increasing dietary protein [55] and particularly red meat [56]. Patel *et al.* also showed a reduction of urinary indoxyl sulphate when comparing healthy volunteers following a vegetarian diet to volunteers following an unrestricted diet [57]. As it has been linked to an increased risk of cardiovascular diseases [58], some dietary strategies have emerged to lower the level of indoxyl sulphate, such as lowering protein intake or probiotic supplementation with a *Lactobacillus* strain [59,60]. Considering those results, we hypothesize a modulation of the gut microbiota of HRPC in favour of both an increase of deleterious bacterial families and/or a decrease in beneficial ones such as *Bifidobacteriaceae* and

Lactobacillaceae. However, all these three metabolites were no longer found significant when the model was adjusted on dietary fibre intake, indicating that red and processed meat intake only is not sufficient to explain the observed associations. As fruits, vegetables, fibres, calcium intake and other nutritional intakes were significantly different between HRPC and NRPC in this cohort, reflecting different overall dietary patterns, we assume that all these factors have a varying impact on the observed microbiota alteration. Our results suggest that not all nutritional factors have the same weight on the observed metabolic perturbations. When the model was adjusted on calcium intake, only a few changes were observed compared to the original model, while adjusting on fibre intake only two discriminant metabolites, *N*-acetyl-leucine and tyrosine, remained significantly different between NRPC and HRPC.

Despite the difficulty of deciphering the complexity of diet multifactor effects based on food questionnaires, a specific effect of red/processed meat has to be considered since similar differences in cinnamoylglycine and indoxyl sulphate levels were observed in the preclinical experiment conducted on rats. As rat diets were adjusted for fat, protein and calories but not for iron/red meat, this argues for a specific effect of red meat consumption on cinnamoylglycine and indoxyl sulphate levels. Microbiota sequencing carried out on these animals strengthen the hypothesis of a dysbiosis induced by high red meat consumption. In fact, as revealed by β-diversity results and differential abundances analysis, alterations in bacterial composition were observed both at the OTUs, genus and phylum levels. In this study a great decrease of genus associated with health benefits such as *Bifidobacterium and Parasutterella* was observed [61,62]. This may explain some of the differences observed in abundance of microbiota-derived metabolites. In accordance with these findings, both a decrease of *Bifidobacterium* and an increase in *Limosilactobacillus* have already been

described in other studies. In particular Zhu *et al.* have reported an increase in *Limosilactobacillus* with high red meat diet compared to a control casein diet [63,64], while Le Leu *et al.* reported a decrease of *Bifidobacterium* with high red meat diet [65].

In the present work, we observed a tremendous increase of MACs in the preclinical experiment, and particularly of lipid peroxidation related species such as DHN-MA. In accordance with this finding, many other animal studies demonstrated an increase in lipid peroxidation products when a heme-rich diet was given [66–68]. Moreover, the modulation of mercapturate profile by heme iron was recently highlighted by Jamin *et al.* who observed an increase of both known and unknown MACs levels in urine of rats submitted to a diet combining hemin and polyunsaturated fatty acids [17]. In the present study, we observed the same phenomenon with a more complex food matrix, closer to real life human diets. The high number of MACs altered by the HRM diet underlined the diversity and complexity of products generated by heme induced lipid peroxidation. Only three MACs could be formally annotated, highlighting the current lack of knowledge about these compounds and underlining the poor availability of analytical standards and spectral data. However, this mercapturome alteration has not been observed in the human study, especially for lipid peroxidation derived MACs, probably because of a less contrasted situation due to a more balanced diet, containing, among other things, more antioxidants and a lower proportion of red meat. However, it should be noted that MACs can also originate from other compounds such as polyphenols [69]. This could explain the observed decrease of some mercapturic species in HRPC. The absence of an increased level of urinary lipid peroxidation products observed in HRPC may be linked to the calcium content of the diet which is known to reduce heme-induced lipid peroxidation [70,71]. In the preclinical study, the diet calcium content is reduced while in the human cohort calcium consumption is in the range of the nutritional

recommendations for NRPC and slightly below for HRPC. Finally, it is worth noting that a urinary mercapturic acid profile is transitory [68] and highlights only a recent exposure to electrophilic substances, therefore strongly depending on the sampling time slot. In the human study, red and processed meat consumption was assessed as an average of repeated 24h dietary records (at least 6) over the two years preceding the urine sampling and therefore reflects long-term dietary habits rather than intakes occurring just before the urine sampling. Conversely to MACs profile, gut microbiota alterations may be less transitory and would be least sensitive to the sampling time.

The originality of this work lies in several points. First, the vast majority of LC-MS metabolomic studies aiming to highlight markers of red and processed meat consumption have been performed using positive ionisation, in particular for the detection of biomarkers such as acyl-carnitines or carnosine. Acyl-carnitines have been widely described as biomarkers of red and processed meat consumption [72–74]. In a recent study, Wedekind *et al.* strengthened the link between red and processed meat consumption and increased urinary acyl-carnitines by a metabolomic analysis combining samples from both an intervention and an observational study [74]. Khodorova *et al.* confirmed the origin of acyl carnitine as well as other compounds such as phenylacetyl glutamine or 1-methylhistidine using ^{15}N-labelled bovine meat in an intervention study [73]. In our work, the use of negative ionisation allowed highlighting other metabolites that may not be efficiently detected in the positive ionisation mode. This could increase the knowledge on biomarkers of red and processed meat consumption. Second, the human study compared three groups of participants differing by their usual consumption of red and processed meat and matched for several individual characteristics, which reinforced the capacity to highlight differences in urinary metabolome while reducing inter-individual variability. The extensive

characterisation of participants from the large prospective NutriNet-Santé cohort, especially regarding dietary data, also allowed to consider numerous nutritional and lifestyle parameters as potential confounding factors. In addition, participants in the NutriNet-Santé cohort display some particularities including: (i) they are predominantly women, and (ii) they overrepresent high socio-professional categories. Therefore, they are more likely to display a healthier nutritional profile, with consequently a lower consumption of red and processed meat, compared to the general French population [75,76]. In spite of this, we were still able to highlight biomarkers of consumption in this study, while characteristics of the Nutrinet-Santé study mentioned above may have limited our ability to detect further significant differences in the urinary metabolome. Finally, our findings on the human cohort were supported by similar trends observed in a preclinical intervention experiment in rats, which allowed strengthening our hypothesis of a gut microbiota alteration induced by high red meat diet. However, considering the hypothesis of a red meat-induced microbiota dysbiosis, this type of study would, in the future, highly beneficiate from microbiome sequencing data in human together with urinary metabolome analysis. Unfortunately, no sample is available to carry out a metagenomic study on the subjects investigated in this work. Further work is foreseen for assessing both metabolomic and microbiome.

In conclusion, this work demonstrated a urinary metabolome modification associated with high red and processed meat consumption in both a human cohort and a preclinical experiment on rats. To the best of our knowledge, some metabolites such as cinnamoylglycine or HPLA have never been described as being associated with red/processed meat consumption. This particular metabolic signature led us to assume a microbiota dysbiosis induced by red meat consumption, a hypothesis reinforced by microbiota sequencing data on rats. The ability of high red meat diet to induce the

production of electrophilic and reactive compounds was demonstrated in rats but not in humans. Nevertheless, in view of the results of the preclinical experiment it would be worth studying mercapturic profile in human after an intervention study with a controlled diet. By providing new markers of consumption, this work may help to better understand the impact of red and processed meat consumption on health and in particular the potential deleterious effect on gut microbiota.

Conflict of interest All authors declare that they have no potential conflict of interest.

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Data sharing — Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval. Researchers from public institutions can submit a collaboration request including information on the institution and a brief description of the

project to collaboration@etude -nutrinet-sante.fr. All requests will be reviewed by the steering committee of the NutriNet-Santé study. A financial contribution may be requested. If the collaboration is accepted, a data access agreement will be necessary and appropriate authorizations from the competent administrative authorities may be needed. In accordance with existing regulations, no personal data will be accessible.

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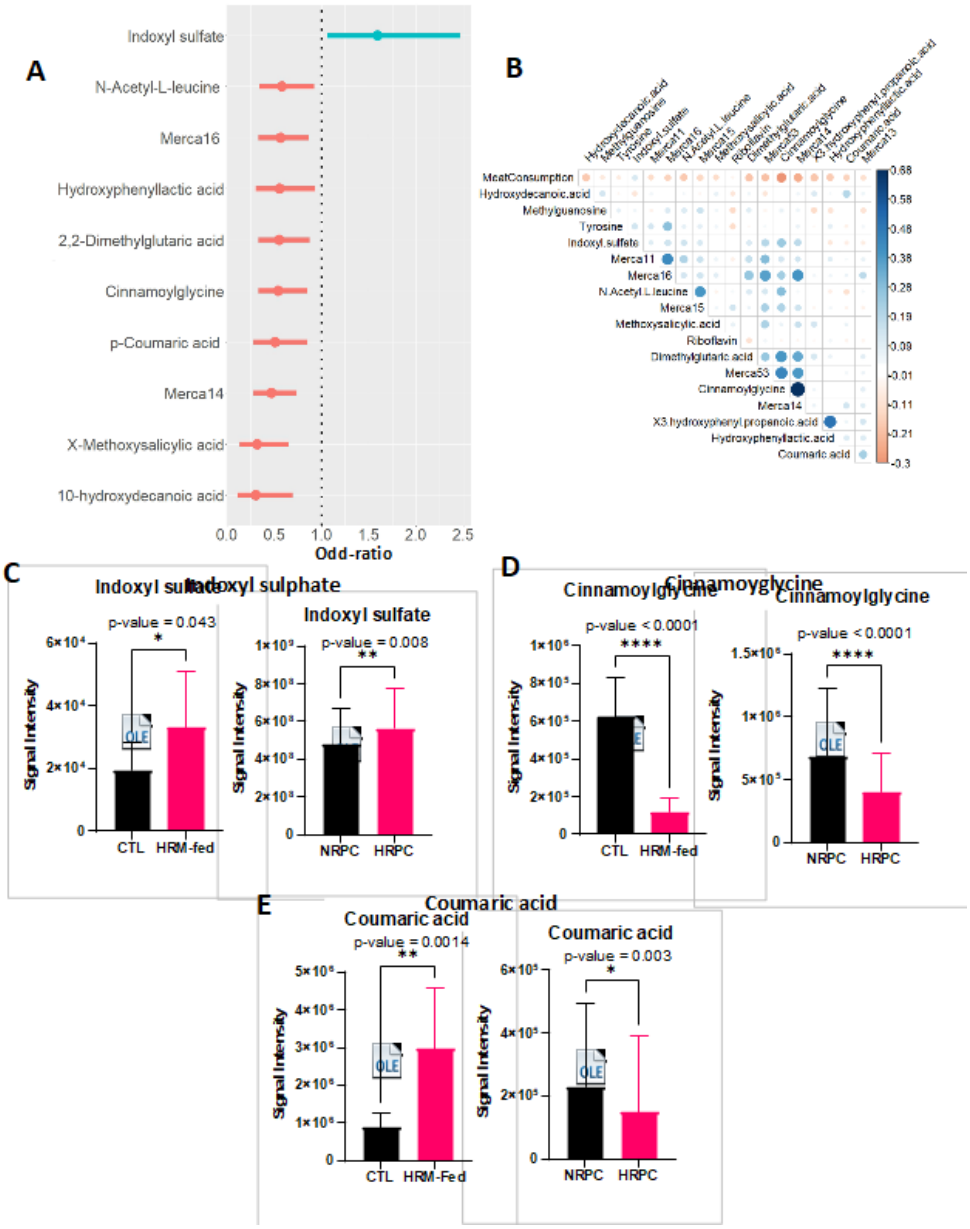

Figure 1 Odd-ratios of selected metabolites in the non-nutritionally adjusted model (A) Correlation matrix between red meat consumption and metabolite signal intensity for all metabolites selected in every calculated model (B) Indoxyl sulphate signal intensity measured in both animal experiment and human cohort samples (C) Cinnamoylglycine signal intensity measured in both animal experiment and human cohort samples (D) *p*-coumaric acid signal intensity measured in both animal experiment and human cohort samples (E)

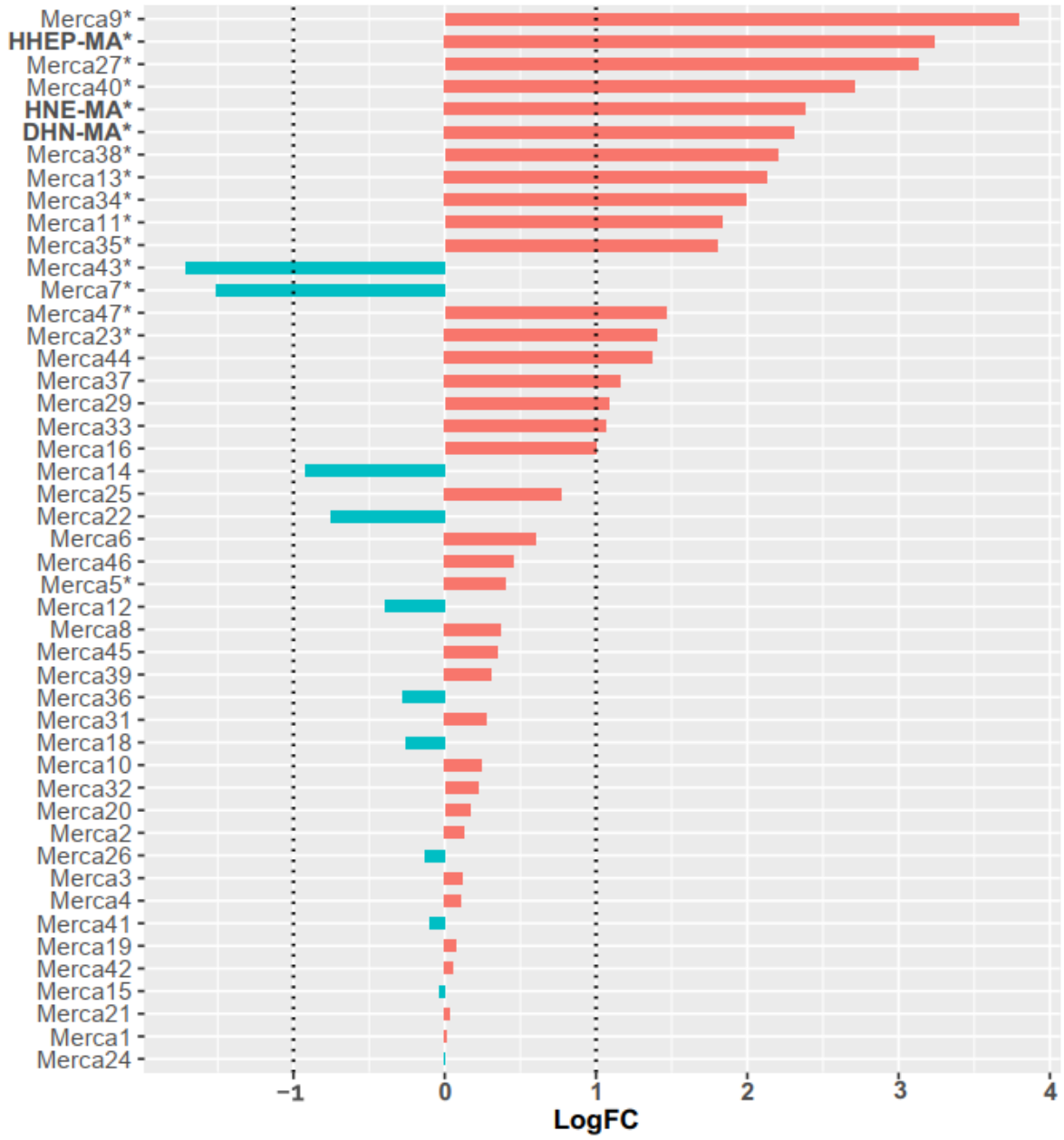

Figure 2 Log2 fold change of all 47 MACs detected in the animal experiment. « * » correspond to MACs which ViP is > 1 in the OPLS-DA model

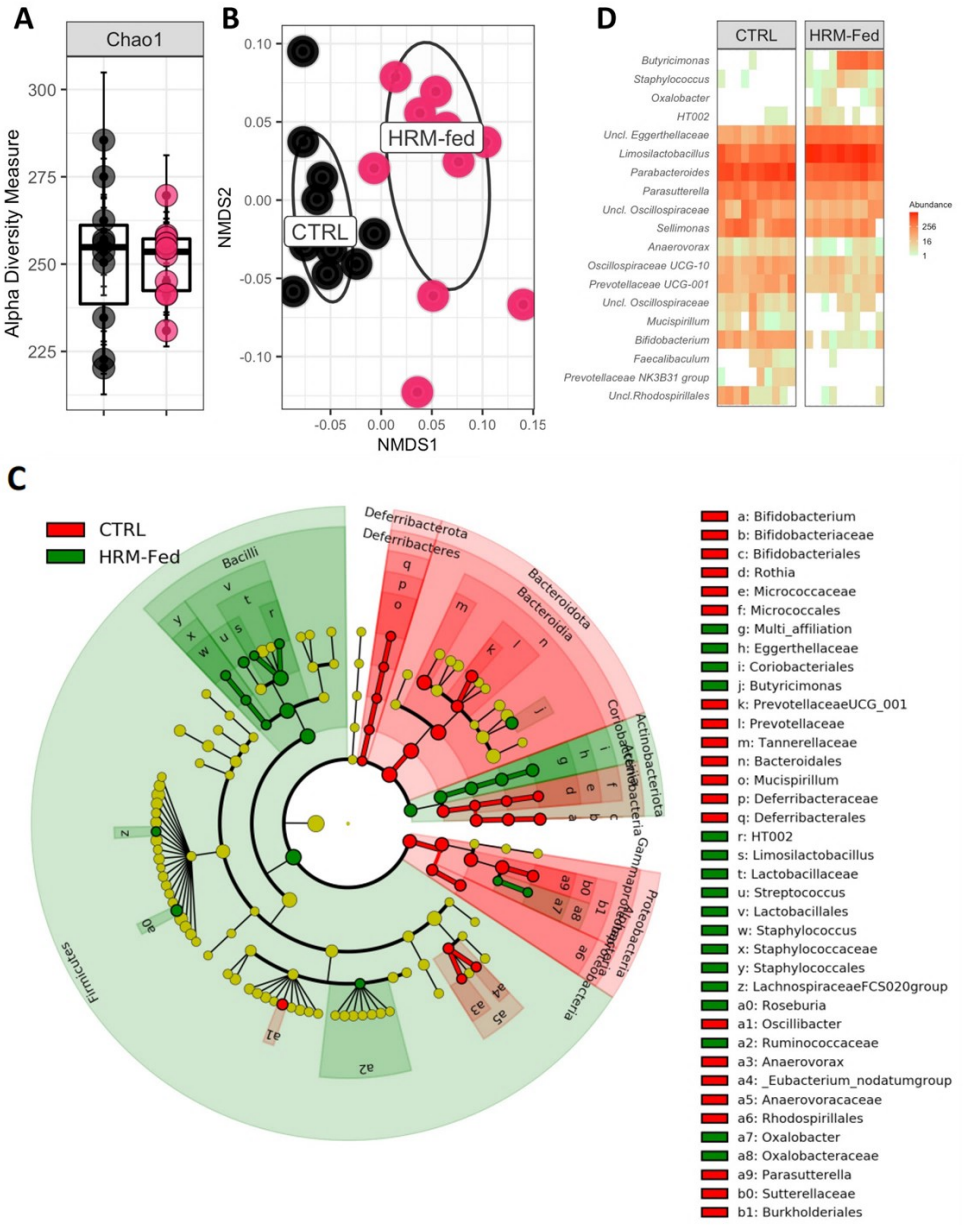

Figure 3 α-diversity determined by Chao 1 index (A) β-diversity based on UniFrac distances (B) Circular cladogram generated from LEfSe analysis showing the most differentially abundant taxa enriched in microbiota from rats fed with control (red) or red meat (green) diets (C) Significant changes observed at the genus level between microbiota of rats fed with CTL or HRM diet (D)

Table 1. Lifestyle and dietary characteristics of the study population. Values are in mean ± SD or n(%)

	High red/processed meat consumers (n=80)	Non red/processed meat consumers (n=80)	Randomly selected participants (n=80)
Age	50 ± 13	49.9 ± 12.8	50.2 ± 12.7
Kcal	1968.3 ± 453.7	1951.6 ± 414.9	1941.3 ± 415.3
R.meat-Proc.meat[a]	123.4 ± 30.5	0 ± 0	65.9 ± 40.7
BMI	24.9 ± 4.7	21.5 ± 2.8	23.6 ± 4.6
Alcohol (g/day)	11.7 ± 12.6	4.6 ± 7.8	9.1 ± 13.1
Heme iron (g/day)	1.8 ± 0.9	0.1 ± 0.5	1.3 ± 1.4
Survey nb	7.5 ± 2	7.2 ± 1.8	7.5 ± 2.1
Protein(g/day)	88.1 ± 21	78.3 ± 20	63.5 ± 21
Polyphenols[b]	1344.3 ± 567.8	1710.5 ± 755.9	1274.3 ± 448.8
Ca (mg/day)	911.9 ± 275.5	993.6 ± 342.3	945.7 ± 249.5
Fibre (g/day)	19 ± 6	29 ± 9.6	20.4 ± 6
Sum vitamins[c]	212.7 ± 63.1	259.9 ± 112	206.9 ± 58.1
Sum veg-fruits[d]	419 ± 196.5	618.4 ± 320.2	439.6 ± 181.9
Physical activity			
Low	25 (31.25)	37 (46.83)	26 (32.5)
Moderate	31 (38.75)	30 (37.97)	38 (47.5)
High	24 (30)	12 (15.18)	16 (20)
Educational level			
Primary	12 (15)	11 (13.75)	12 (15)
Secondary	17 (21.25)	11 (13.75)	12 (15)
Superior	51 (63.75)	58 (72.5)	56 (70)

[a] Sum of red and processed meat consumption in g/day

[b] Sum of polyphenol consumption in mg/day

[c] Sum of Vitamin C, E, Selenium and zinc consumption per day

[d] Sum of vegetable and fruit consumption in g/day

Graphical abstract

In this study, the impact of high red meat consumption was investigated in a general population cohort whose results were supported by an in vivo experiment. Using a non-invasive urine analysis, a probable modification of microbiota coupled to an increase of oxidative stress was demonstrated and confirmed using an animal model. This study highlighted changes occurring in general population and provides clues to explore the adverse effects associated with high red meat consumption.