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Consumption of boiled, but not grilled, roasted or barbecued beef modifies the urinary metabolite profiles in rats.

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Abbreviations : **DKP**, diketopiperazine, **DMU**, dimethyluric acid, **EIC**, extracted ion chromatogram, **4GDBA**, 4-guanidinobutanoic acid, **IC-DA**, Independent Components-Discriminant Analysis, **1-MIA**, 1-methylimidazoleacetic acid, **7-MG**, 7-methylguanine, **3-MH**, 3-methylhistidine, **NOC**, *N*-nitroso compounds, **QC**, quality control.

Keywords : animal study, biomarkers, chemometrics, meat cooking method, urinary metabolomics

Abstract

Scope : The consumption of processed meat is associated with increased risk of chronic diseases, but determining how the exposure to specific cooking processes alter the metabolome is an analytical challenge. We aimed to evaluate the impact of four typical cooking methods for beef (boiling, barbecuing, grilling, and roasting) on the urinary metabolite profiles in rats, using a non-targeted approach.

Methods and results

Male Wistar rats (n = 48) were fed for three weeks with experimental diets containing either raw or cooked (boiled, barbecued, grilled and roasted) beef. A control group was fed with milk proteins. The 24h-urines were analyzed using LC-MS. The consumption of boiled meat led to the specific excretion of di- and tri-peptides (aspartyl-leucine, glycyl-aspartate and aspartyl-prolyl-threonine) and a cyclo-prolyl-proline ($p < 0.001$). No singular metabolite specifically associated with the groups “grilled”, “roasted” and “barbecued” meat was observed.

Conclusion

Urinary metabolite profiles of rats fed boiled beef were clearly distinct from those of rats fed with raw, grilled, roasted or barbecued beef. The specific metabolites included the products of non digested proteins and might be useful as potential intake biomarkers of this meat cooking method.

1. Introduction

Meat is an important contributor to protein intake in industrialized countries ^[1]. Many studies associate the consumption of red and processed meats to an increased risk of colorectal cancer, a higher subsequent risk of developing type 2 diabetes and cardiovascular diseases ^[2-4]. Identifying biomarkers of meat consumption according to their type (white/red, processed/unprocessed) is thus of interest to improve the exposure assessment in cohort studies ^[5-8].

It is well established that cooking meat at high temperatures is conducive to the formation of heterocyclic aromatic amines and polycyclic hydrocarbons, which have potentially mutagenic and carcinogenic effects in humans. Studies have attempted to detect the excretion of these specific molecules and/or their conjugates after intake of meat cooked with various techniques ^[9-10], but their detection remains an analytical challenge: first, owing to their extremely low concentration; and second, due to their rapid excretion (within 12 h after meat intake). Moreover, these compounds are also formed when grilling chicken or fish, and thus are not specific to red meat. Consequently, it is crucial to highlight novel metabolites associated with different cooking processes of red meat. Recent studies applying the non-targeted metabolomic approach to *in vitro* digests of various types of processed meat have permitted identification of specific molecules for smoked and fermented meat ^[11-12]. However, analysis of human biological fluids is needed to confirm their ability to predict the intake of these products.

The cooking processes may also alter the digestibility of meat protein, depending on the cooking intensity ^[13]. Although meat is highly digestible, some undigested parts that reach the colon are fermented, which may result in the production of molecules, including potentially genotoxic products such as *N*-nitroso compounds (NOC). We have shown that long cooking times (boiling) decreased beef protein digestibility slightly but significantly ^[14].

In our previous study of urinary metabolites in humans after an acute intake of raw or fully cooked beef, we were unable to distinguish between a moderate or intensive cooking process [15]. This could be ascribed to the feeding protocol (subjects ingesting a single portion of 130 g of meat), which may not have been sufficient to detect urinary biomarkers related to the cooking intensity in the eight hours following ingestion. In the present study, we aimed to evaluate the impact of four typical cooking methods of bovine meat (boiling, barbecuing, grilling, and roasting) as compared to raw meat on the urinary metabolite profiles in rats exposed to cooked meat for three weeks. The untargeted metabolite profiling applied to the samples could help elucidate novel red meat-associated metabolites, as well as specific compounds associated with each cooking process, which may be used to evaluate the exposure to meat cooked with different processes in epidemiological studies.

2. Experimental Section

2.1. Meat and cooking processes

The cooking techniques for the bovine meat used in the experimental diets were chosen in order to be representative of human eating habits. The cooking processes applied to the beef used for experimental diets are described in Table 1.

Table 1. Characteristics of cooking processes applied to the beef samples.

Cooking method	Internal T, °C	Total cooking time, min	Details
Grilling	60-64	5-7	Meat slices (1 cm thick) cooked on a plancha at 240 °C. Meat slices were turned at 2 min of cooking time.

Barbecuing	60-64	15	Meat slices (1 cm thick)
Roasting	60-62	35-40	Meat pieces of 500 g cooked in an oven at 180°C.
Boiling	100	210	Meat pieces (2 cm x 2 cm) cooked in boiling water.

2.2. Animals and experimental design

This study was conducted in accordance with French guidelines on animal experimentation and validated by the Ethics Committee in Animal Experiments of INRAE Jouy-en-Josas (Comethea, registration number: 12-089). Detailed information on the study design can be found in the published parent study ^[14]. Male Wistar rats (n = 48) were adapted to the diet containing milk proteins as the only source of dietary proteins over a period of one week. Animals were then randomly split into six groups of eight rats and were fed for three weeks with experimental diets. The experimental diets contained either raw meat or meat cooked according to four different methods. One experimental diet did not contain any meat, and the rats continued to receive milk proteins (control group). A group consuming raw meat was also used as control for cooked meat groups. The 24h-urines were collected at the end of the adaptation period and after three weeks of experimental diets.

2.3. Urine sample preparation

One hundred micro-litres of each thawed 24h-urine sample were diluted with 900 µl of milliQ water/acetonitrile (90/10, v/v), filtered using 0.2 µm Phenex-RC Syringe Filters with a cellulose membrane (Phenomenex, Le Pecq, France), transferred to a sealed glass autosampler vial and either kept at -20°C or stored at 4°C if used within one to two days for LC-MS analysis. Quality

control (QC) samples were prepared by combining small aliquots of all samples. Analytical samples were analysed in random order, interspersed after every 10 injections with QC sample and blank (acetonitrile/water 10/90, v/v) injections to monitor instrument performance and sample stability.

2.4. Mass spectrometry analyses

All organic solvents were LC-MS grade from Sigma-Aldrich, and all aqueous solutions were prepared using purified distilled water from Millipore Milli-Q system. The authentic standards of 7-methylguanine, 4-guanidinobutanoic acid, 1-methyl-4-imidazoleacetic acid, carnitine, and deoxycarnitine were purchased from Sigma-Aldrich.

LC-MS analysis was performed on an Acquity H-Class system coupled to a Q-TOF Synapt G2 Si instrument (Waters Corporation, Milford, MA). The urine samples were analysed using two chromatographic techniques: reverse-phase chromatography (RP) and hydrophilic interaction chromatography (HILIC) in positive electrospray ionization mode (ESI⁺). The RP chromatography was performed on an Acquity CSH C18 column (2.1 × 100 mm; 1.7 μm bead size; Waters). Column temperature was 40°C and the eluents A and B were 0.01% formic acid in water and 0.01% formic acid in acetonitrile, respectively. The gradient was run at 0.4 mL/min and consisted of an isocratic elution for 0.5 min of 5% B, and then B was increased at a linear rate to 95% at 10 min, then re-equilibrated for 1 min with 5% B and held at 5% B until 16 min. For HILIC, an Acquity BEH Amide column (2.1 × 100 mm; 1.7 μm bead size; Waters) was used. Column temperature was 45°C and the eluents were in acetonitrile:20 mM of pH 3.5 ammonium formate (50:50, v:v; eluent A) and acetonitrile:20 mM of pH 3.5 ammonium formate (90:10, v:v; eluent B). The HILIC gradient was run at 0.6 ml/min and consisted of isocratic elution for 2.5 min of 100% B, then B decreased at a linear rate to 0 % in 10 min, then re-equilibrated for 1 min with 100% B and held at 100% B until 15 min. Injection volume was 2 μL for both RP and HILIC.

The MS source conditions were as follows: capillary voltage at 3.0 kV; cone voltage at 20 V; source offset 20 V. The MS data were collected from 50 to 1100 Da in centroid mode at 0.2 sec/scan using leucine-enkephalin as a lock mass to correct mass accuracy. The MS spectra were recorded in the centroid mode at 0.2 sec/scan. MS/MS experiments were performed on selected $[M+H]^+$ ions using argon as the collision gas and collision induced dissociation (CID) parameters are reported in Table 2.

2.5. LC-MS data processing and analysis

Raw data from Synapt G2 Si were pre-processed using MarkerLynx (MassLynx V4.1, Waters) to obtain a list of detected features characterised by m/z and retention time (R_t). An extracted ion chromatogram (EIC) window of 0.03 Da, an intensity threshold of 500 counts, a noise elimination level (standard deviation above background) of 8 and a retention time window of 0.2 min were used. Isotope peaks were removed by MarkerLynx. Integrated peak areas for the detected features were transferred to Matlab version 7.6.0 (Mathworks Inc.) chemometrics analysis by Independent Components-Discriminant Analysis (IC-DA) and for statistical evaluation of the data (ANOVA). The identification of the biomarker candidates was accomplished by exact mass measurement and comparison of MS/MS spectra with authentic standards and/or earlier published data or searching databases (Metlin, Human Metabolome Database, KEGG).

2.6. Data Analysis

Each spectral data matrix was Pareto-scaled and analysed by Independent Components-Discriminant Analysis (IC-DA)^[16]. IC-DA is a supervised version of Independent Components Analysis (ICA)^[17], which has been successfully applied in a previous study^[15]. A brief description of this method includes mention that before applying ICA, the spectral data matrix is augmented with information on the data, namely the group membership of each sample. Hence, the extracted independent components (ICs) are oriented in a way such that the group

information is taken into account, leading to the best possible discriminating ICs. The projection of the samples on the ICs can be plotted, and lead to the visualization of possible group discriminations.

Once discriminant ICs have been extracted, the original m/z variables found significant on S-plots can be found. An ANOVA of each of these individual variables helps find the variables which can be used alone to discriminate one group versus the others. Independent Components-Discriminant Analysis models were calculated with in house routines written in Matlab R 2008a. The ICA algorithm used was the Joint Approximate Diagonalization of Eigenmatrices (JADE) [18-20].

The predictive performance of the IC-DA models was tested by group permutations and by comparing the F-distributions of the corresponding Wilks' lambdas to the F-value obtained for the true groups. It was verified whether this "true" F-value was higher than all the F-values calculated for the permuted groups with empirical P values of 0, thus confirming the validity of the obtained model.

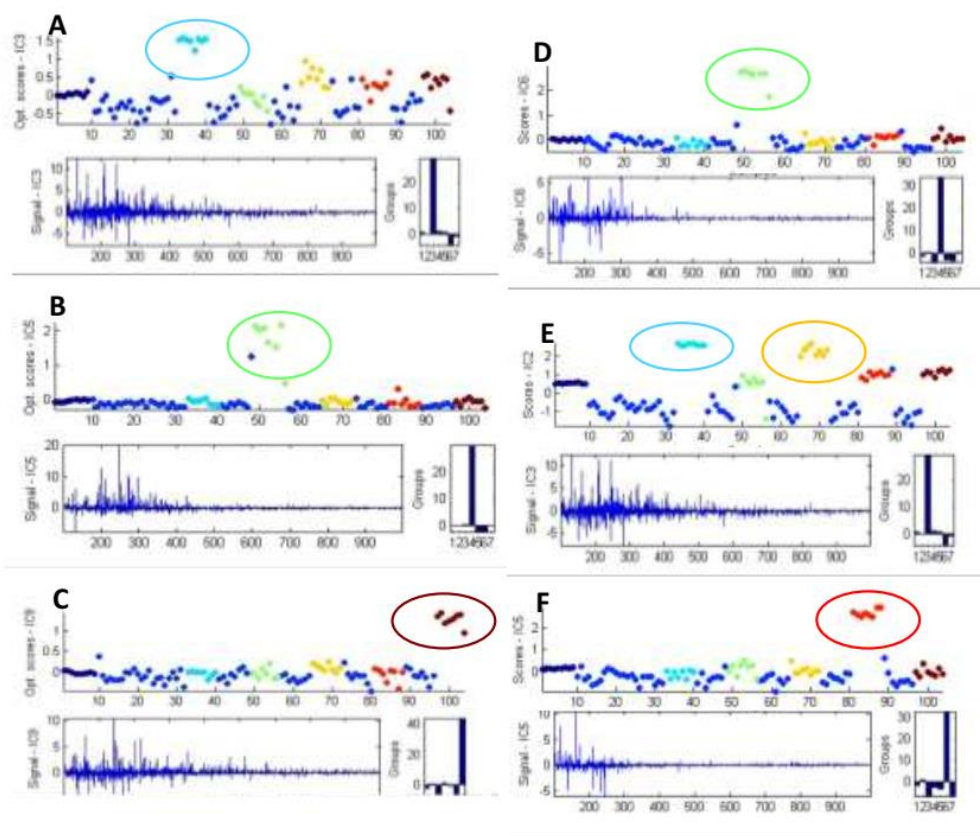
The S-Plot was used to select the most relevant variables for the separation of groups. The selection of discriminant variables was based on correlations and covariances, and contributions to the ICs that were greater than +/- 3 standard deviations of all the contributions to each component.

3. Results

IC-DA was applied separately to mass spectral data obtained by HILIC and RP chromatography. Six groups were included in IC-DA: control diet, raw meat, boiled, barbecued, grilled and roasted meat, depending on the period "before meat consumption" and "after meat consumption". The groups raw meat, boiled and roasted meat were separated along the IC3, IC5 and IC9, respectively, after source signal extraction by IC-DA from the data obtained by RP chromatography (Fig. 1, A-C).

Figure 1. Score-plots obtained by IC-DA applied to MS data obtained by RP (A, B, C) and HILIC (D, E, F).

■ raw meat
 ■ boiled meat
 ■ roasted meat
 ■ barbecue meat
 ■ grilled meat



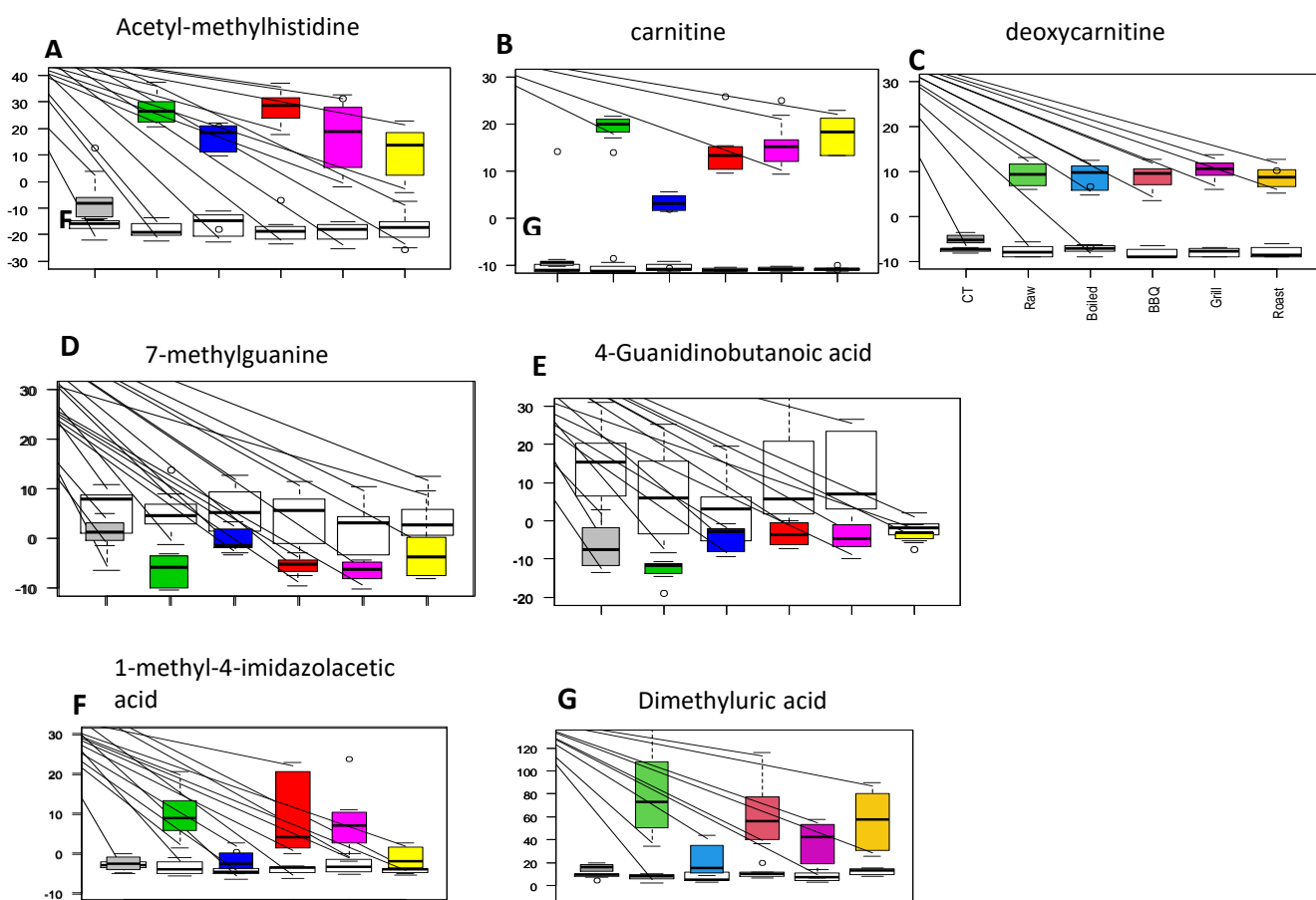
The data obtained by HILIC also permitted separation of the groups “boiled” meat in IC6 and “grilled” meat along IC5 (Fig. 1, D and E, respectively), whereas IC2 permitted the separation of “raw” and “barbecued” meat groups (Fig. 1, F). Thus, the groups from “raw” and “boiled” meat could be distinguished from other groups based on the data from both polar and non-polar compounds, whereas “grilled” and “roasted” with only one type of compound. It is interesting to note that only the group “barbecued meat” was not separated alone along any IC.

After IC-DA, we further analyzed the variables with the highest intensities on each IC by a univariate statistical analysis, ANOVA, in order to detect whether the individual variables permitted separation of the groups. It is important to be aware that this method is informative, only, and might not deliver the expected results. Indeed, IC-DA is a multivariate method, and therefore, the biomarkers highlighted on the ICs enable a discrimination of the samples when used altogether. However, there is no evidence that they will be discriminant when used in a univariate fashion. Among 420 features subjected to ANOVA, 43 were significantly elevated or decreased after consumption of meat or were related to specific meat cooking methods (p -value <0.05). Amongst them, we were able to identify 14 with the confidence level 1 or 2, according to the Metabolomics Standards Initiative ^[21]. The detailed information on metabolite identification is reported in Table 2. The compounds annotated with confidence level 3 and unknown compounds are shown in Supplementary materials Table S1.

The intensity of 15 identified metabolites is presented in Fig. 2 and 3. A significant effect of meat consumption on the changes of carnitine ($p<0.001$), deoxycarnitine ($p<0.001$), and acetyl-methylhistidine ($p=0.025$) were found. Compared to the control group (milk) and to baseline, these metabolites were excreted more in all groups of rats having consumed meat, independent of the cooking method (Fig. 2, A-C).

Figure 2. Relative signal intensities of metabolites related to the consumption of beef meat in urines of rats.

■ raw meat
 ■ boiled meat
 ■ roasted meat
 ■ barbecue meat
 ■ grilled meat



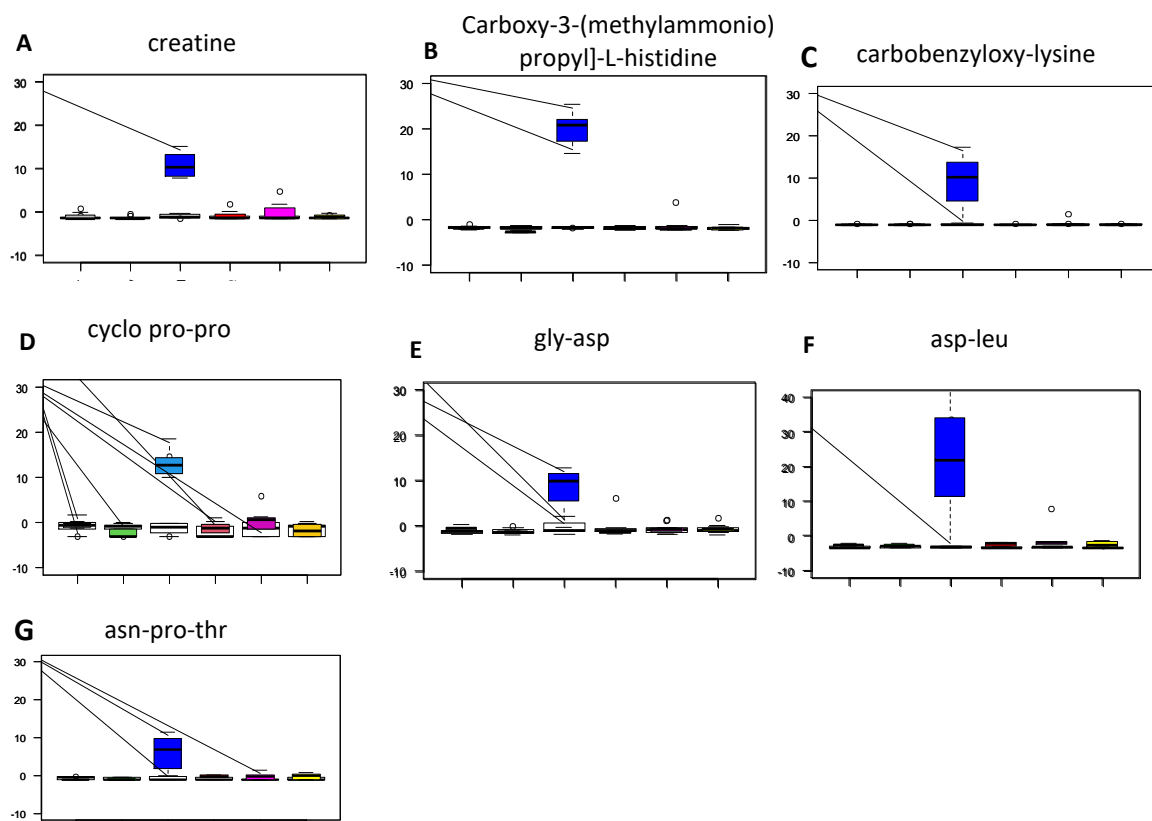
The intensity of two purine-related compounds, 7-methylguanidine (7-MG) and 4-guanidinobutanoic acid (4GDBA), was altered after feeding with meat-containing diets. Indeed, the excretion of 7-MG decreased after meat consumption in all groups as compared to the baseline (Fig. 2, D), and this decrease was more significant for the group of rats that consumed raw meat ($p=0.05$). The excretion of 4GDBA tended to be lower in the group “raw meat” as compared to the control group ($p=0.057$), whereas in the groups fed cooked meat the levels were similar to those of the control group (Fig. 2, E).

We were not able to detect any individual metabolites specifically associated with the groups “barbecued”, “grilled” and “roasted” meat. Even if the higher excretion of 1-methylimidazoleacetic acid (1-MIA) was observed after consumption of “barbecued” and “grilled” meat, its signal intensity was also elevated in urine from the “raw” meat group (Fig. 2, F). Similarly, dimethyluric acid and 2-methylbutyrylcarnitine were detected at higher levels in the urines of “barbecued”, “grilled” and “roasted” meat groups, but also for “raw” meat (Fig. 2, G, H).

In contrast, we were able to identify seven urinary metabolites associated to the consumption of boiled meat (Fig. 3). Both at baseline and in the urines from the groups other than “boiled” meat, these metabolites were not detected, or were present only as traces. These compounds included creatine ($p < 0.001$) and derived product of histidine ($p < 0.001$) and lysine ($p < 0.001$; Fig 3, A-C), and several di- and tripeptides: a cyclic peptide cycloprolyl-proline, ($p < 0.001$; Fig. 3, D) dipeptides aspartyl-leucine ($p < 0.001$) and glycyl-aspartate ($p < 0.001$, Fig 3. E-F), and a tripeptide asparagyl-prolyl-threonine ($p < 0.001$; Fig. 3, G).

Figure 3. Relative signal intensities of compounds related to the consumption of boiled beef meat in urines of rats.

■ raw meat
 ■ boiled meat
 ■ roasted meat
 ■ barbecue meat
 ■ grilled meat



4. Discussion

The aims of the present study were to identify the urinary metabolites related to the consumption of bovine meat (cooked and uncooked) in rats, and to evaluate metabolic signatures representative of four typical cooking methods of bovine meat (boiling, barbecuing, grilling, and roasting) in comparison to raw meat. This was achieved using an LC-MS non-targeted metabolite profiling. We were able to identify the biomarkers for general bovine meat consumption, and also to differentiate between metabolic profiles related to the cooking types. Specific metabolites were identified.

4.1. Metabolites of bovine meat intake

Carnitine and deoxycarnitine were highly excreted in urine of rats consuming meat, independent of the cooking method applied. It is well established that the consumption of red meat, rich in carnitine, leads to an elevated urinary excretion of trimethylamine-containing compounds [22]. In addition, numerous studies have demonstrated the association between the intake of different animal proteins (red meat and poultry) and urinary excretion of 3-methylhistidine (3-MH). Although 3-MH was not altered in our study, its derived compound, N-acetyl-3-MH, was elevated in urines after meat consumption. N-acetyl 3-MH is one form in which 3-MH may normally be excreted. In adult humans, the acetylated form represents about 5% of urinary 3-MH, whereas in rats, more than 85% of urinary 3-MH is excreted as its acetylated derivative [23-24]. The fact that we observed an elevated urinary concentration of acetyl 3-MH, and not of 3-MH after meat consumption is thus probably due to the specificity of the rat metabolism.

Surprisingly, we observed a decrease in the excretion of 7-MG after the consumption of meat-containing diets. The 7-MG is one of the most abundant DNA methylation metabolites observed in normal human biofluids. In two epidemiological studies in healthy populations [25-26], higher urinary 7-methylguanine levels were considered the marker related to the intake of meat, fish and other protein-rich foods, whereas a decreasing trend of 7-methylguanine levels was associated with “healthy” meal habits (lower animal protein intake, higher vegetable content). Red meat, rich in guanine, methionine, choline, folates and vitamins of the B group, increases the endogenous formation of N-nitroso compounds (NOCs). The consumption of diets rich in red meat have been previously associated with increased levels of guanine methylated adducts in the colon and rectal epithelium [27-29], whereas they could not be detected in urine, probably due to their low concentration [30]. As guanine is nitrosable and considered a NOC precursor, we suggest that the lower excretion of 7-MG may result in NOCs production.

However, in our study, NOCs did not appear to be discriminant biomarkers of meat and/or cooking process. In accordance, a study by Billson et al. ^[31], aiming to examine the associations between diet and biomarkers of exposure in colorectal tissue, did not establish any relationship between red or processed meat consumption and 7-MG levels in colorectal mucosa.

The guanidines conjugated with carboxylic acids have also been previously identified as biomarkers of meat consumption. For instance, guanidinoacetate has been reported as a marker of animal protein intake ^[6, 32], whereas urinary levels of guanidinobutanoate permit differentiation between whey and cooked beef protein intake in rats, higher levels being observed when consuming cooked beef ^[33]. In our study, the levels of 4GDBA in urine after cooked meat intake were similar to the control group.

4.2. Boiled meat

The group “boiled meat” was clearly distinguished from the other groups due to the higher excretion of numerous metabolites, suggesting that the consumption of boiled meat could lead to pronounced changes in the urinary metabolic profile. Previous studies have associated urinary metabolites with compounds formed in meat during high temperature cooking, focusing on heterocyclic amines ^[9a,b], or during meat processing like smoking or fermentation ^[11, 34]. Interestingly, in our study, we identified two molecules that are generally neoformed in meat upon cooking, and three molecules that are not directly related to the thermal treatment.

For instance, we identified the appearance of two modified amino acids, N-2-carbobenzyloxy-lysine and (2-(3-carboxy-(methylammonio)propyl)-histidine), which may be formed in proteins due to the Maillard reaction ^[35]. We also found an increase of a proline-containing dipeptide in its cyclic form. The cyclic dipeptides (diketopiperazines, DKPs) are formed under heating conditions of organic matter containing proteins, and they are found in different beverages and foods in which they are responsible for taste and flavor ^[36, 37]. The DKPs

have already been detected in cooked beef, and the longer cooking times increase the number of DKPs produced as well as their concentration. Consistent with our results, stewing beef for six hours resulted in the formation of 10 cyclic dipeptides at higher concentrations, whereas grilling for 10 min yielded only four at relatively low concentrations [37].

The consumption of boiled meat also led to the accumulation of two other dipeptides and one tripeptide in urine. Several dipeptides such as anserine and carnosine have been well-described as meat intake biomarkers [4, 5, 7, 8, 38, 39]. It was recently shown that the intake of collagen when ingesting red meat increased the urinary excretion of dipeptides containing proline, prolyl-hydroxyproline and ile/leu-hydroxyproline [8]. These molecules, which are related to muscle collagen metabolism, were validated as biomarkers for red meat [8]. The peptides that we observed in urine of rats fed boiled meat contained aspartate or asparagine, which may have originated from less-digested sequences.

Indeed, we have shown previously that cooking beef at a higher temperature for a longer period of time increased exogenous protein flux in the ileum and tended to decrease protein digestibility in humans [40]. Concurrently, we also found that in rats, protein from boiled meat was less digestible compared to other cooking methods such as grilling or barbecuing [14]. Additionally, static *in vitro* studies have shown a decrease of myofibrillar protein digestibility by gastric and pancreatic enzymes for long cooking times. Since the digestibility of protein was moderately decreased with boiling in our previous study in rats [14], the daily consumption of this product may have resulted in a higher production of specific dipeptides by colon bacteria, followed by their absorption by the colonic epithelium that we could detect at the urinary level. Although there is little data to document the absorption of dipeptides in the colon, the presence of the peptide transporter PEPT1 has been shown in the colon of healthy rodents, especially in the distal part, together with an uptake functionality [41]. In other words, the fact that lower protein digestibility in our study was associated to an increased urinary excretion of some

dipeptides in urines may reflect a difference in peptide production in the colon. Another hypothesis is that these dipeptides were more present in the small intestinal lumen after the ingestion of boiled beef and absorbed at this level

4.3. Grilled, roasted and barbecued meat

Urinary profiles did not permit identification of metabolites specific to grilling, roasting and barbecuing. We did find, however, an alteration in the levels of methyl urates 1,4-methyl imidazoleacetic acid (MIA) and dimethyluric acid (1,3 DMU), and 2-methylbutyroylcarnitine, in these groups. 1,4-methyl imidazoleacetic acid showed a higher excretion level in grilled and barbecued groups, as well as after consumption of raw meat, whereas dimethyluric acid and 2-methylbutyroylcarnitine were elevated in all groups except boiled. That the excretion of these compounds was also altered after the consumption of raw meat does not permit their association with the cooking method. At the same time, they could not be considered as metabolites related to general beef intake, because the groups “boiled” and “roasted” showed 1-MIA levels were similar to those of the control. Furthermore, the excretion of dimethyluric acid and 2-methylbutyroylcarnitine did not change following the consumption of “boiled” meat.

The main limitation is that we could identify less than half of compounds that were significantly different between groups. This is a common limitation in untargeted metabolomic studies. Another limitation is that the study was realized in the rat model, but on the other hand it is also a strength since the mid-term nutritional intervention could be strictly controlled. This permitted to detect the metabolites exclusively related to beef intake. Also, the application of an untargeted metabolomics approach allowed to identify new metabolites related to meat cooking method. However, a human study would be warranted to validate whether these metabolites predict the consumption of boiled beef.

In conclusion, this study pioneeringly associates urinary, non-targeted metabolic profiles to the consumption of bovine meat cooked with four typical methods. We characterized seven molecules (including di- and tripeptides and the modified amino acids) excreted specifically after the consumption of boiled meat. These observations are consistent with the values of protein digestibility, the lowest being observed for boiled meat. This suggests that metabolites produced from colon fermentation result in specific signatures in urine. For the meat cooking processes that did not affect protein digestibility (grilling, roasting and barbecuing), no specific signature was significantly observed at the urinary level.

Authors contributions

N.V.K. conducted the metabolomics analysis, analyzed the data and wrote the manuscript; D.J.R.B. carried out the chemometrics analyses and contributed to the discussion; S.P. was involved in metabolomics analysis and contributed to the metabolite identification and discussion; C.C. and N.L. contributed to the discussion; M.O. conducted the animal experiment; C.G. conceived and designed the animal experiment and contributed to the discussion; all authors read and approved the final manuscript.

The authors declare no conflict of interest.

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Table 2. Urinary metabolites related to the consumption of beef in rats identified at MSI confidence level 1 and 2 [21].

RP/HILIC	Experimental m/z	Suggested ion	Elemental composition	MS/MS fragment ions	Collision energy, eV	MSI MI level	Suggested metabolite	P-value
RP	166.0734	[M+H] ⁺	C ₆ H ₈ N ₅ O	149.0465, 124.0513, 107.0246, 96.0563, 79.0300	25	1	7-methylguanine	P _{group} =0.0082 P _{period} <0.001 P _{groupxperiod} =0.0057
HILIC	146.0933	[M+H] ⁺	C ₅ H ₁₂ N ₃ O ₂	128.0796, 111.0553, 100.0769, 87.0461, 86.0606, 68.0415	25	1	4-Guanidinobutanoic acid	P _{group} =0.0243 P _{period} <0.001 P _{groupxperiod} =0.0090
RP	271.1398	[M+H] ⁺	C ₁₁ H ₁₉ N ₄ O ₄	253.1302, 229.1292, 166.0480	20	2	2-[3-Carboxy-3-(methylammonio)propyl]-L-histidine	P _{group} <0.0001 P _{period} <0.0001 P _{groupxperiod} <0.0001
RP	195.1134	[M+H] ⁺	C ₁₀ H ₁₅ N ₂ O ₂	180.0406, 151.0885, 150.0892, 121.0317, 98.0607, 70.0655	25	2	Cyclo (prolyl-proline)	P _{group} <0.0001 P _{period} <0.0001 P _{groupxperiod} <0.0001

HILIC	141.0668	[M+H] ⁺	C ₆ H ₉ N ₂ O ₂	123.0578, 113.0847, 96.0701, 95.0618	20	1	1-Methyl-4- imidazoleacetic acid	P _{group} <0.0001 P _{period} <0.0001 P _{groupxperiod} <0.0001
RP/HILIC	212.0937	[M+H] ⁺	C ₉ H ₁₄ N ₃ O ₃	194.0829, 170.0849, 153.0590, 135.0486, 124.0812, 109.0706, 96.0627, 83.0551	20	2	Acetyl-3- methylhistidine	P _{group} <0.001 P _{period} =0.0017 P _{groupxperiod} =0.0025
RP/HILIC	132.0721	[M+H] ⁺	C ₄ H ₁₀ N ₃ O ₂	90.0455, 87.3298	20	2	creatine	P _{group} <0.001 P _{period} <0.001 P _{groupxperiod} <0.001
HILIC	162.1136	[M+H] ⁺	C ₇ H ₁₆ N ₃ O	103.0324, 85.0233	25	1	carnitine	P _{group} <0.001 P _{period} <0.001 P _{groupxperiod} <0.001
HILIC	146.1184	[M+H] ⁺	C ₇ H ₁₆ N ₂ O	87.0447, 60.0809	20	1	deoxycarnitine	P _{group} <0.001 P _{period} <0.001 P _{groupxperiod} <0.001
RP	331.1605	[M+H] ⁺	C ₁₃ H ₂₃ N ₄ O ₆	313.1448, 271.1392, 226.1167, 217.1294, 200.1046, 175.1194,	20	2	Pro-thr-asn	P _{group} <0.001 P _{period} <0.001 P _{groupxperiod} <0.001

				166.0984, 158.0834, 116.0697, 70.0646				
RP	191.0531	[M+H] ⁺	C6H11N2O5	145.0608, 70.0290	15	2	Gly-asp	P _{group} =0.0009 P _{period} <0.001 P _{groupxperiod} =0.0002
RP	247.1294	[M+H] ⁺	C10H19N2O5	201.1220, 184.0959, 155.1157, 132.1015, 86.0961	20	2	Asp-leu	P _{group} =0.0003 P _{period} <0.001 P _{groupxperiod} <0.001
HILIC	197.0673	[M+H] ⁺	C7H9N4O3	154.0617, 152.0437	20	2	Dimethyluric acid	P _{group} <0.001 P _{period} <0.001 P _{groupxperiod} <0.001
RP	281.1485	[M+H] ⁺	C14H21N2O4	239.1394, 194.1175, 178.0975, 154.0873, 150.0986, 130.0869, 110.0598, 84.0805	25	2	N- benzyloxycarbonyl- lysine	P _{group} <0.001 P _{period} <0.001 P _{groupxperiod} <0.001

RP	246.1699	[M+H] ⁺	C ₁₂ H ₂₄ NO ₄	187.0942, 85.0289, 144.1017	15	2	2-Methylbutyrocarnitine	$P_{\text{group}} = 0.0097$ $P_{\text{period}} < 0.001$ $P_{\text{group} \times \text{period}} < 0.001$
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